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<b>(54) Title:</b> PROCESS FOR THE PREPARATION OF AQUEOUS DISPERSIONS OF PARTICLES OF WATER-SOLUBLE POLYMERS AND THE PARTICLES OBTAINED  <b>(57) Abstract</b>  A process for the preparation of crosslinked water-soluble polymer particles comprising, combining an aqueous polymer solution comprising a water-soluble polymer, particularly a polysaccharide, and an aqueous medium, and an oil medium so as to form an emulsion of droplets of the water-soluble polymer, and adding to the emulsion a crosslinking agent capable of crosslinking the water-soluble polymer so as to form crosslinked water-soluble polymer particles, the particles formed thereby and aqueous dispersions thereof. The invention also encompasses a method comprising, administering to a patient in need of treatment an aqueous suspension of the water-soluble polymer particles.		

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**PROCESS FOR THE PREPARATION OF AQUEOUS  
DISPERSIONS OF PARTICLES  
OF WATER-SOLUBLE POLYMERS  
AND THE PARTICLES OBTAINED**

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**Background of the Invention**

**(A) Field of the Invention**

20           This invention relates to the preparation of aqueous dispersions of water-soluble polymer particles and to the particles obtained from such a process.

**(B) Description of the Prior Art**

25           Aqueous dispersions of water-soluble polymer particles are difficult to manufacture. It is particularly difficult to control particle size and shape and to make such particles reproducibly. Aqueous dispersions of water-soluble polymer particles that have been available prior to this invention are, in general, not satisfactory. Previously, such particles are generally prepared by adding a  
30           crosslinking agent to an aqueous polymer solution under agitation. Agitation serves to break down the crosslinking polymer to give the desired particle size. To break down the crosslinked and crosslinking polymers, sufficient mechanical stress to cause shearing and disruption of particle integrity is required. This concurrent  
35           agitation-crosslinking method gives an undesirably broad distribution of particle sizes and shapes, and produces undesirable particle

fragments. Additionally, there is substantial variability in the resulting particle populations between production runs.

A particularly undesirable aspect of the heterogeneous particle population produced by this method is that it is difficult or impossible to "clean up" or fractionate the heterogeneous population to yield a homogeneous population of particles having the same size and shape. A homogeneous population of particles is particularly desirable where the particles are to be delivered by injection through a narrow gauge syringe needle.

Other processes for preparing aqueous dispersions of crosslinked water-soluble polymer particles involve methods that are of a proprietary nature. Such particles, however, are either not available in the quantities wanted, their physical parameters are not suitable for the application or their properties cannot be varied systematically. Moreover, a full range of polymer compositions and sizes has not been available.

Water-soluble particles have various utilities. There are three basic types of water-based polymers:

(a) solution polymers-these comprise dispersions of individual polymer molecules. The viscosity of the solution depends upon the molecular weight and concentration of the polymer; the higher the molecular weight and the concentration, the higher is the viscosity of the solution. The viscosity of the solution limits the use of these solution polymers to low molecular weights and low concentrations. This is especially true where the use involves delivery of particles via injection through a narrow gauge syringe needle.

(b) latexes-these comprise colloidal dispersions of polymer particles, each of which comprises hundreds or thousands of polymer molecules. The viscosity of the latex depends upon the interactions between the colloidal particles and is independent of the molecular weight of the polymer. Thus, latexes often combine low viscosities with high polymer concentrations. Moreover, the mechanism and kinetics of emulsion polymerization favor the preparation of high-molecular-weight polymers with rapid rates of



polymerization. Aqueous dispersions of crosslinked water-soluble polymers can be prepared by inverse emulsion or inverse suspension polymerization of monomer mixtures containing a crosslinking monomer, e.g., a mixture of acrylamide and methylene-bis-acrylamide. However, this method is limited to polymers that can be prepared by radical chain polymerization. There remains a need to prepare particles of water-soluble polymers that are derived from natural sources.

(c) Water-reducible or water-dispersible polymers—these polymers have a degree of dispersion intermediate between that of solution polymers and that of latexes. The viscosity of these intermediate samples depends upon the relative degrees of solution polymer and latex polymer character. As with the latexes, this method is limited to polymers that can be prepared by radical chain polymerization.

Polysaccharides are naturally occurring biopolymers that exist in a highly viscous liquid state in animal tissues, where they readily react with proteins to form glycosaminoglycans or proteoglycans.

Biocompatible and non-biodegradable particles that are non-cytotoxic, non-carcinogenic, non-inflammatory, non-pyrogenic, and non-immunogenic, are needed to provide a solution to the long felt and unfulfilled need for an improved composition useful for soft tissue augmentation to treat congenital abnormalities, acquired defects or cosmetic defects.

Homogeneous populations of such particles would be particularly useful for soft tissue augmentation by surgical implantation or, preferably, delivery to the desired site by convention injection through a narrow gauge syringe needle.

It would be particularly desirable to use such particles to treat urinary incontinence and vesicoureteral reflux, for correction of wrinkles and other skin defects, or to serve as a general augmentation or replacement composition or as a scaffold material in soft or hard tissues such as breast, lip, penis, bone, cartilage, and tendon.

There are no reports in the literature until now of how to prepare the corresponding hydrophilic gel microspheres of sodium alginate and sodium hyaluronate.

Thus, there is a need for aqueous dispersions of water-soluble polymer particles of a relatively narrow size distribution with defined physical characteristics, and for a process for preparing such a dispersion and recovery of particles in any desired quantity at reasonable cost.

#### 10 Summary of the Invention

It is a primary object of this invention to provide a process for the preparation of aqueous dispersions of water-soluble polymer particles not having the problems of the prior art.

It is a further object to provide a process for the preparation of aqueous dispersions of water-soluble polymer particles of a narrow size distribution.

It is another object to provide water-soluble polymer particles having substantially uniform shape.

It is yet another object of the invention to provide a process for the preparation of aqueous dispersions of water-soluble polymer particles wherein the particles recovered have a size distribution such that the greater majority of the particles have a size less than 212 micrometers.

It is a further object of the invention to provide a process for the preparation of aqueous dispersions of water-soluble polymer particles wherein at least about 80% of the particles are of a size less than 150 micrometers.

A feature of the invention is that the water soluble polymers are dissolved in an aqueous medium and then combined with an oil medium to form a water-in-oil emulsion.

Another desirable feature of the invention is that the water-soluble polymer particles can be readily extruded through a hypodermic syringe needle.

An advantage of the invention is that the water-soluble polymer particles formed thereby are produced in a relatively

narrow distribution of particle sizes useful for soft tissue augmentation. Moreover, the particles can be produced in large quantities at reasonable cost as well as in small quantities in the laboratory.

5 Of advantage also is that the water-soluble polymer particles of the invention may be used as a vehicle to deliver encapsulated drugs and other medications *in vivo*. Another advantage is that the polymer particles are injectable, and thus easily and inexpensively administered to a patient.

10 Additionally, the particles have other uses, such as in cements and adhesives, thickeners of latex paints, substitutes for microcrystalline cellulose, nutrient media, and substitutes for agar gels with better-defined gel sizes.

15 The invention relates to a process for the preparation of aqueous dispersions of water-soluble polymer particles which comprises,

(a) emulsification of an aqueous solution of a water-soluble polymer in a continuous oil medium using a water-in-oil emulsifier to form a water-in-oil emulsion of droplets;

20 (b) crosslinking of the droplets formed by the emulsion; and

(c) inversion of the crosslinked polymer-in-oil droplets into water (or otherwise transferring the polymer) to give a dispersion of crosslinked water-soluble particles in water.

25 The invention further relates to water-soluble polymer particles formed by this process and to aqueous dispersions thereof. Desirably, the water-polymer particles of the invention are substantially homogeneous in size and shape, and range in size from 10 to 250 micrometers, desirably less than 212 micrometers in  
30 diameter, more desirably, less than 150 micrometers in diameter, even more desirably less than 50 micrometers.

#### Detailed Description of the Invention

35 The present invention relates to a process for preparing crosslinked water-soluble (hydrophilic) polymer particles, the

polymer particles formed by the process, and aqueous dispersions and pharmaceutically acceptable dispersions of the polymer particles. The invention also relates to therapeutic methods of using of the polymer particles.

5           The present invention provides biocompatible and non-biodegradable particles that are substantially non-cytotoxic, non-carcinogenic, non-inflammatory, non-pyrogenic, and non-immunogenic, and which lack other unwanted humoral or cellular responses. Desirably the particles are also possess sufficient long  
10 term stability of size, shape, rigidity and composition to have utility as implant materials. A further desirable characteristic of the particles of the invention, useful for implant purposes is that they are relatively inert and do not rapidly degrade *in vivo*. A particularly advantageous feature of the particles of the invention is that they are  
15 easily injectable. Desirably the crosslinked water-soluble polymer particles prepared according to the invention are substantially homogeneous in size, and generally of a size less than 212 nm in diameter, and at least 80% of the particles are spherical. More desirably at least 90% of the particles are spherical. Still more  
20 desirably, at least 80% of the particles are within one standard deviation of the mean or average size of the particles. Such particles provide a solution to the long felt and unfulfilled need for an improved composition useful for medical treatments, such as soft tissue augmentation to treat congenital abnormalities, acquired  
25 defects or cosmetic defects.

Examples of congenital defects treatable with the particles of the invention include without limitation, hemifacial microsomia, malar and zygomatic hypoplasia, unilateral mammary hypoplasia, pectus excavatum, pectoralis agenesis and velopharyngeal  
30 incompetence secondary to cleft palate repair or submucous cleft palate.

Examples of acquired defects include without limitation post surgical, post traumatic and post infectious defects such as depressed scars, subcutaneous atrophy, acne pitting, linear

scleroderma with subcutaneous atrophy, saddle-nose deformity  
Romberg's disease and unilateral vocal cord paralysis.

Cosmetic defects include without limitation glabellar  
frown lines, nasolabial creases, circumoral geographical wrinkles,  
5 sunken cheeks and mammary hypoplasia.

The homogeneous populations of particles produced by  
the process of the invention are useful for soft tissue augmentation  
involving delivery to the desired implantation site by conventional  
injection through a narrow gauge syringe needle (such as a 20, 21 or  
10 22 gauge needle), although they are also useful for surgical  
implantation and other delivery methods such as endoscopically.

A desirable use of the particles of the invention is to  
treat urinary incontinence, vesicoureteral reflux, glottic  
insufficiency, and gastroesophageal reflux, and for correction of  
15 wrinkles and other skin defects, or to serve as a general augmentation  
or replacement composition or as a scaffold material in soft or hard  
tissues such as breast, lip, penis, bone, cartilage, and tendon. When  
the particles of the invention are administered to a patient to serve as  
scaffold material, the particles facilitate the migration and infiltration  
20 of fibroblasts and related cells. The large free spaces within the  
microsphere of the present invention, particularly polysaccharide  
microspheres, allows cells to infiltrate into and through the bead.  
Additionally, the large surface area of the microspheres promotes  
attachment and growth of infiltrating cells. Still further, the  
25 scaffolding material is gradually degraded and absorbed by the host.  
Optionally, when the microspheres of the present invention are used  
as scaffolding material, bioactive agents can be crosslinked, coupled  
or otherwise attached using methods well known in the art, to  
provide localized cellular stimuli. Examples of such bioactive agents  
30 include growth factors and cytokines such as fibroblastic growth  
factor, endothelial growth factor, interleukins, platelet derived growth  
factor, tissue necrosis factor, hormones, cell adhesion peptides, etc.  
Accordingly, when the microspheres of the present invention are  
used as scaffolding material, colonization of the site by cells is  
35 facilitated. and into the site of injection of the

A particularly advantageous feature of the invention is that process parameters such as the composition of the oil phase employed, the emulsifying agents selected, temperature, pH, crosslinking time and washing, can be adjusted and precisely controlled in small scale synthesis, and then readily and reproducibly duplicated when scaled up to full production runs to achieve superior microparticles having desirable characteristics such as substantially uniform size and shape, excellent physical and chemical stability, and elastic properties that permit easy extrusion through small gauge syringe needles.

The process of preparing crosslinked water-soluble polymer particles comprises, dissolving a water-soluble polymer in water or aqueous buffer to provide the desired concentration of the polymer in solution. The aqueous polymer-containing solution is then added, in suitable amount to provide the desired concentration of the water-soluble polymer, to an oil phase which includes a water-in-oil emulsifying agent. The mixture is agitated to form an emulsion containing polymer droplets. The polymer droplets are crosslinked in situ by a crosslinking agent resulting in the formation of crosslinked polymer particles. Following crosslinking, the polymer in oil emulsion is then inverted in an excess of water to provide an aqueous dispersion of crosslinked polymer particles. The particles are then recovered from the aqueous dispersion.

Any water-soluble polymer, natural or synthetic, can be used in the practice of the invention provided that (1) the viscosity of an aqueous solution thereof is low enough that such can be broken down into droplets during the emulsification step, and (2) it contains functional groups that can serve as sites for reaction with a crosslinking agent. Generally, solutions with viscosities as high as  $10^4$  -  $10^5$  cps can be broken down into droplets by the usual methods of emulsification.

Water soluble polymers that will be found satisfactory in the practice of the invention include, for example, proteins, polysaccharides, peptidoglycans (heteroglycan chains comprising alternating units of N-acetylglucosamine (GlcNAc) and N-

acetylmuramic acid (MurNAc) linked to various peptides), glycoproteins (proteins to which carbohydrate chains are attached), proteoglycans (proteins to which glycosaminoglycan chains are linked), teichoic acids, lipopolysaccharides, synthetic hydrophilic  
5 polymers and mixtures thereof. Desirably, the particles of the invention are formed from a single water-soluble polymer.

Examples of desirable hydrophilic polymers include but are not limited to, natural polysaccharides such as hyaluronic acid, sodium alginate, chondroitin sulfate, celluloses, chitin, chitosan,  
10 agarose, dextrans, xanthans, dermatan sulfate, keratin sulfate, amylose, emulsan, gellan, curdlan, amylose, carrageenans, amylopectin, dextrans, glycogen, starch, heparin sulfate and limit dextrans and fragments thereof; synthetic hydrophilic polymers such as poly(ethylene oxide), poly(vinyl alcohol), and poly(N-vinyl  
15 pyrrolidone); and proteins such as bovine serum albumin, and human gamma globulin. Hyaluronic acid and sodium alginate are polysaccharides that are biocompatible and biodegradable in human tissue, and are non-cytotoxic, non-carcinogenic, non-inflammatory, non-pyrogenic, and non-immunogenic; therefore, in the form of gel  
20 microspheres, they are good candidates for delivery of drugs.

Various crosslinking agents will be found satisfactory. The one most desirable will depend somewhat upon the particular water-soluble polymer being used. Importantly, the water-soluble polymer and cross-linking agent will both have functional groups  
25 reactive with one another. The selection of an appropriate cross-linking agent can readily be accomplished by those skilled in the art. Examples of crosslinking agents which will be found satisfactory include pentaerythritol-tris-[beta (N-aziridiny)-propionate], divinylsulfone, glutaraldehyde, p-toluene sulfonic acid, carbodiimides, diepoxides, ammonium persulfate. Calcium can also  
30 be used in some cases. For example, the calcium ion is known to crosslink water-soluble polymers that contain carboxyl groups. Nevertheless, this reaction forms ionic bonds, rather than covalent bonds. Ionic bonds may be broken down by a change in external  
35 conditions, e.g., by chelating agents. On the other hand, covalent

bonds are stable in the presence of chelating agents. Thus, the most preferred crosslinking agent for use in the practice of the invention is one that forms covalent bonds with the water-soluble polymer, rather than ionic bonds.

5           The crosslinking agent is necessarily added, in some cases, to the aqueous polymer solution prior to emulsification as will be made more clear hereinafter. In other cases, however, the crosslinking agent can be added to the dispersion of water-soluble droplets in the oil phase. In some cases, the crosslinking agent can  
10 even be added to the inverted emulsion. The order of addition of the crosslinking agent will depend not only upon the particular polymer and crosslinking agent chosen but also upon the rate of the crosslinking reaction. An important consideration is that the rate of crosslinking not be such that it is competitive with the emulsification.  
15 Desirably the crosslinking reaction is slow enough to permit complete emulsification of the aqueous and oil phases. Optionally, the crosslinking agent may be added to the aqueous polymer solution, and that the pH of the solution adjusted to suitably slow down the rate of crosslinking prior to such solution being added to, and emulsified  
20 with, the oil phase. Subsequently, the pH is adjusted to provide the desired rate of crosslinking reaction.

In some cases, it may be desirable to add a catalyst to initiate the crosslinking reaction. This will depend somewhat upon the particular water-soluble polymer selected, as well as the  
25 crosslinking agent. The selection of any particular catalyst, in any case, can be readily done by those skilled in the art. Examples of catalysts that can be used in the practice of the invention include p-toluene sulfonic acid and hydrochloric acid. It may be desirable, in some cases, to provide heat as a catalyst.

30           The oil phase (i.e. the non-aqueous solvent phase) can be any inert hydrophobic liquid which can be readily separated from the dispersion of water-soluble polymer particles. Those which will be found suitable in the practice of the invention include toluene, o-xylene, and isooctane; however, in general, any hydrocarbon can be  
35 used as the oil phase liquid. Of concern, however, is that the



crosslinking agent should not be soluble in the oil phase. Neither should the water-soluble polymer solution be soluble or miscible with the hydrocarbon used as the oil phase. Desirably, the hydrocarbon should also be substantially pure, although mixtures of hydrocarbons may be used. Desirably, the hydrocarbon is substantially non-volatile. Those skilled in the art will, it is believed, be able to choose a suitable hydrocarbon for use in the practice of the invention. Although not critical to the invention disclosed herein, the hydrocarbon chosen, from a practical standpoint, should be low in cost.

In order to emulsify the water soluble polymer phase into the oil phase to give a water-in-oil emulsion, an emulsifying agent of the water-in-oil type is used in the amount of from about 0.010 to about 10.0 weight percent of the oil phase. Any commonly used water-in-oil emulsifying agent can be used in the practice of the invention, for example, hexadecyl sodium phthalate, sorbitan monostearate, metal soaps and the like. Nevertheless, how well a given emulsifier works in any particular case depends upon the polymer solution to be emulsified, the composition of the oil phase, and the means of emulsification. The emulsifier must function to stabilize the water-in-solvent system of the invention sufficiently to permit controlled crosslinking of the hydrophilic polymers to yield particles that are substantially homogeneous in size and shape, i.e. approximately 80% of the particles are within one standard deviation of the mean size, and at least approximately 95% of the particles are spherical. Desirable emulsifiers have low HLB (Hydrophile-Lipophile Balance) values, generally HLB values of less than approximately 8, preferably less than approximately 6 i.e., they are water-in-oil emulsifiers. Those skilled in the art can readily determine, which water-in-oil emulsifier to use in any given case.

The crosslinking reaction is controlled to some extent by the particular reactants involved, the concentration of the reactants, and by the pH. The ratio of the weight of the water-soluble polymer to that of the crosslinking agent will depend upon the particular polymer being used as well as the crosslinking agent. This ratio can

vary from about 0.2 to about 200, preferably from about 1 to about 10. The extent of crosslinking of the polymer droplets is generally controlled by a specified time at room temperature. In any particular case, the most suitable reaction time can be readily determined by those skilled in the art. In some cases the crosslinking reaction can be stopped by addition of an alcohol such as methanol or isopropanol, the hydroxyl groups of such reacting with the functional groups of the crosslinking agent. The pH can be adjusted as desired, using, for example, a base such as ammonium hydroxide, or an acid such as acetic or hydrochloric acid.

The extent of agitation desired of the emulsion during droplet formation will depend not only upon the recipe of the reactants involved in any particular case but, also upon the size of the particles desired. For example, as will become more apparent hereinafter, toluene containing SPAN 60 emulsifying agent, i.e. sorbitan monostearate having a HLB of 4.7, which is available from ICI Americas, can be used to make microscopic-size particles, e.g., 50 micrometers diameter, or submicroscopic-size particle, e.g., 0.2 micrometers diameter, according to the concentration of the SPAN 60 and the agitation. A low SPAN 60 concentration with vigorous agitation will give larger particles; a high concentration with vigorous agitation will give smaller particles. Different types of agitation and mixing equipment, as will be appreciated by those skilled in the art, can be used to obtain particles of a desired size. Desirably, the agitation used to stir the reactants, and particles resulting therefrom, is sufficient to completely mix the reactants, but less than that which generates mechanical stress forces resulting in shearing.

The separation of the crosslinked particles from the aqueous phase can be accomplished by various known procedures. Generally, after inversion of the polymer particles into an aqueous phase, the oil layer is separated from the aqueous phase, e.g., by sedimentation - leaving an aqueous dispersion of crosslinked polymer particles. Then, the crosslinked water-soluble polymer particles are separated from the aqueous phase by filtration, after which the

particles are washed and dehydrated with methanol. The particles are then dried. This can be done by merely spreading the particles out on a flat surface; however, in the case of an industrial scale operation the particles can be dried in a fluidized bed drier conventionally used  
5 for such a purpose.

The size of particles resulting from the practice of the invention, quite advantageously, can vary over a wide range of sizes, both microscopic and submicroscopic, according to the average droplet size of the emulsion, which depends upon the composition,  
10 type and concentration of emulsifier, as well as the emulsification procedure and conditions.

The particles may be microcapsules, microspheres, or beads. Microcapsules are defined as polymer particles containing one or more encapsulated compositions. Desirably, the encapsulated  
15 compositions are drugs, such as, but not limited by, antigens, antibodies, growth factors, inhibitors, antibiotics, antisense oligonucleotides, antiviral compositions, anti-cancer compositions, therapeutic agents, and other compositions to be administered to a patient, or delivered to a specific site in a patient. Microcapsules are  
20 large enough to be seen by the naked eye. Microspheres, on the other hand, are much smaller particles that generally do not contain encapsulated materials. Moreover, they may require optical microscopy to be seen. Beads are spherical-shaped particles that are large enough to be seen with the naked eye. The limit of visibility of  
25 beads is in the range of from 60-100 micrometers.

Desirably, the polymer particles of the invention are less than 212 micrometers in diameter, more desirably less than 150 micrometers in diameter. Advantageously, the particles formed are substantially homogeneous in size and shape. Another desirable  
30 feature of the water-soluble polymer particles of the invention is that when dispersed in water and injected through a 20 gauge or 22 gauge hypodermic syringe needle, a worm-like thread is formed. Injectability is an important characteristic of the water-soluble microspheres when the particles are used for drug delivery or  
35 therapy.

The invention will be further understood in light of the following non-limiting examples:

**Example 1**

**Preparation of An Aqueous Dispersion of Water-Soluble Polymer Particles In The Shape of Crosslinked Microspheres**

This example shows the preparation of an aqueous dispersion of crosslinked water-soluble particles of sodium alginate in the shape of microspheres. The recipe is set forth in Table I below:

**TABLE I**

**Recipe for Crosslinked Water-Soluble Polymer Particles**

	<u>Ingredient</u>	<u>Parts by Weight</u>
20	Water	100.00
	Sodium alginate water-soluble polymer	7.00
	30% Ammonium hydroxide to adjust pH to 10-11	18 drops
	Toluene	100.00
	SPAN 60 emulsifier	1.00
25	XAMA-7 crosslinking agent	4.00
	Isopropanol dehydrating agent	100.00

The ammonium hydroxide was added to a 5% aqueous solution of sodium alginate containing XAMA-7 pentaerythritol-tris-[beta-(N-aziridiny)-propionate] crosslinking agent to adjust the pH to pH 11. With this crosslinking agent, this pH adjustment is critical to prevent premature crosslinking. The aqueous solution was then emulsified in a continuous toluene phase using SPAN 60 water-in-oil emulsifier to give a water-in-oil emulsion or dispersion of aqueous sodium alginate droplets containing the crosslinking agent. Once the emulsion was formed and the desired droplet size distribution was

achieved, a small amount of acetic acid was added to lower the pH to 7-8. The sodium alginate droplets crosslink rapidly at this lower pH. The crosslinked polymer solution-in-oil dispersion was then inverted in an excess of water, after which the oil phase was separated from the aqueous dispersion to give an aqueous dispersion of crosslinked sodium alginate microspheres.

The emulsifier and conditions of agitation were selected to give the desired droplet size (and hence the desired polymer particle size). This size distribution was controlled, not only by the emulsifier used, but also by the type and intensity of agitation used to prepare the water-in-oil emulsion. The results of these experiments are shown in Table II.

TABLE II

Particle Size Distribution of Aqueous Sodium  
Alginate Dispersions

Run No.	S-48	S-49	S-50	S-51	S-52	S-53	S-54
Initial pH*	8	10-11	10-11	10-11	10-11	10-11	10-11
Final pH**	8	7-8	7-8	7-8	7-8	7-8	7-8
Stirring rate (rpm)	1000	900	800	800	1200	1000	1000
Microsphere Size (%)							
>250 um	13.5	7.1	7.4	5.1	3.9	8.9	12.3
212-250 um	4.1	2.3	8.8	4.2	3.8	2.1	2.8
150-212 um	11.7	11.6	23.5	14.7	11.8	26.9	8.3
<150 um	70.3	79.0	60.3	70.1	80.6	82.2	76.6

\* after addition of ammonium hydroxide

\*\* after addition of acetic acid

Table II shows the polymer microsphere size distribution achieved under various conditions. Substantially all of the particles were spherical, with few offsize smaller spheres or irregularly shaped particles. Thus, the data in Table II demonstrate that crosslinked sodium alginate particles having a spherical shape

and a diameter of less than 150 micrometers can be prepared reproducibly using the XAMA-7 crosslinking agent, by adjusting the pH of the sodium alginate phase with ammonium hydroxide to pH 11, forming the water-in-oil emulsion, and subsequently lowering the pH to 7-8 with acetic acid to initiate rapid crosslinking of the polymers in the droplets to form polymer particles.

Five types of reaction flasks and stirrers were used in the practice of the invention. The first, designated as A, comprised a one-liter round-bottom flask equipped with a TEFLON polytetrafluoroethylene half-moon stirrer. The second, designated as B, comprised a two-liter kettle equipped with a stainless-steel turbine agitator and six baffles angled slightly in the direction opposite to that of the liquid rotation. The third, designated as C, was similar to B, but of one-half-liter capacity and without the baffles. The fourth, designated as D, was similar to C, but equipped with a Glas-Col GKH Stir Tester to control the speed of emulsification despite the varying loads imposed by the emulsification. The fifth, designated as E, was similar to D, but equipped with a Glas-Col GKH Stir Tester and a stainless-steel turbine shaft. This reaction flask C was used in run S-48 through S-54 shown in Table II.

## Example 2

### Microspheres Obtained From Crosslinked Droplets of Sodium Alginate/Methyl Cellulose

In another experiment, 50.0 g water containing 2.25 g dissolved sodium alginate and 0.25 g Methocel K4M methyl cellulose (5% w/w sodium alginate/Methocel K4M) was dispersed in 75.0 g isooctane containing 1.5 g SPAN 85 (sorbitan trioleate, HLB 1.8, ICI Americas) in the one-half liter C kettle and stirred (about 1000 rpm) for about 10 minutes. Then, 5.0 g water containing 1.0 g TWEEN 85 (ethoxylated sorbitan trioleate, HLB 11.0, ICI Americas) was added, and the dispersion was stirred for another 5 minutes. To crosslink the droplets formed by the dispersion, an equivalent amount of the XAMA-7 crosslinking agent or its aqueous solution was added and

allowed to react with the dispersed polymer droplets for 180 minutes. Then, 25 ml isopropanol was added to dehydrate and harden the crosslinked microspheres. The stirring was continued for 10 minutes, after which the mixture separated into a clear supernatant layer and an opaque, white sedimented microsphere layer. The  
5 filtration of the microspheres proved to be difficult; therefore, the supernatant layer was decanted, and the sedimented microspheres were washed twice by stirring overnight in a beaker with 200 ml isopropanol. The microspheres were then filtered on filter paper  
10 without difficulty and dried at room temperature.

### Example 3

#### 15 Effect of pH Control On The Crosslinking of The Water-Soluble Polymer Droplets To Form Microspheres Suitable For Drug Delivery

This example demonstrates the effect of pH control on the crosslinking of the water soluble polymer droplets in the  
20 formation of microspheres suitable for drug delivery. 100 g of an aqueous solution containing 7.00 g sodium alginate and 4.0 g XAMA-7 crosslinking agent were mixed with 18 drops 30% aqueous ammonia to adjust the pH to 11. This solution was then dispersed in 100 g toluene containing 1.00 g SPAN 60 in the 0.5 liter stirred flask  
25 C using the stainless steel turbine agitator for 30 minutes at about 1000 rpm. The formation of the microscopic droplet size of the water soluble polymer in the water-in-oil emulsion was monitored by optical microscopy while the emulsion was being stirred. When the emulsion was judged satisfactory, sufficient acetic acid was added to  
30 decrease the pH of the aqueous phase to 7-8. This mixture was then stirred for about 4-5 hours at room temperature to allow the XAMA-7 to crosslink the sodium alginate droplets to form microspheres. Then, the aqueous liquid layer was separated by decantation. The microspheres were then washed twice with about  
35 200 ml methanol by sedimentation-decantation, then filtered and dried at 75°C.

All of the microspheres obtained, when dispersed in water and injected through a hypodermic syringe, formed worm-like threads indicating the microspheres were suitable for drug delivery.

### Example 4

### Size Distribution of Cross-linked Sodium Alginate Microspheres Obtained

Other dispersions of sodium alginate were prepared using the recipe shown in Table III below to determine particle size distribution:

### TABLE III

### Recipe for Crosslinked Water-Soluble Polymer Particles

	<b><u>Ingredient</u></b>	<b><u>Parts by Weight</u></b>
20	Water	150.00
	Sodium alginate water-soluble polymer	10.50
	pH (controlled by addition of 30% ammonium hydroxide)	10-11
	Toluene	150.00
	SPAN 60 emulsifier	1.50
25	XAMA-7 crosslinking agent	6.00
	pH (controlled by addition of 10% acetic acid)	7-8
	Isopropanol dehydrating agent	150.00

30 161 g of an aqueous solution containing 10.50 g sodium alginate was mixed with sufficient 30% aqueous ammonia to adjust the pH to 10-11. This solution was dispersed in 150 g toluene containing 1.50 g SPAN 60 in the 0.5 liter capacity stirred D flask using the stainless steel turbine agitator, as earlier disclosed, for 30  
35 minutes at 1000 rpm. The droplet size was monitored by optical microscopy while the emulsion was being stirred. When it was deemed satisfactory, sufficient 10% acetic acid was added to lower



the pH of the aqueous phase to 7-8. This mixture was then stirred for 6 hours at room temperature to allow the XAMA-7 to crosslink the sodium alginate droplets. Following crosslinking, 150 g isopropanol was added to dehydrate the beads. The stirring was continued for  
 5 another 30 minutes. Afterwards, the liquid layer was separated by decantation. The microspheres were washed twice with about 200 ml methanol by sedimentation-decantation, then filtered, and dried at 75°C.

10 The size distributions for the microspheres obtained in each case is given in Table IV.

Table IV

15 Particle Size of Aqueous Sodium Alginate Dispersions

Run. No.	S-56	S-57	S-58
Reaction flask	E	E	E
Reaction time (hr)	6	6	6
20 Stirring rate (rpm)	1000-800	800	1000
Microsphere Size (%)			
a. >250 um	10.6	6.4	6.4
b. 212-250 um	3.1	4.5	1.8
c. 150-212 um	7.0	15.4	9.3
25 d. <150 um	79.0	73.6	82.6
Spherical morphology of microspheres			
before pH 7-8*	good	good	good
after pH 7-8**	good	good	good
small particles***	++	+	++
30 irregular particles***	++	++	+

\* after addition of ammonium hydroxide

\*\* after addition of acetic acid

\*\*\* +- few particles; ++ - more particles

All of the microspheres obtained in these experiments, when dispersed in water and injected through a hypodermic syringe, formed the desirable worm-like threads.

5

### Example 5

#### Effect of XAMA-7 Crosslinking Agent At Various Concentrations In Aqueous Solution At pH 7

10

This example is to show the effect of the crosslinking agent at various concentrations in the aqueous polymer solution on the formation of microspheres.

15

Three problems were encountered in the first experiments with the XAMA-7 crosslinking system: (1) The microsphere distribution was broader than desired; (2) The proportion of sodium alginate in the crosslinked polymer was too low and that of the XAMA -7 crosslinking agent was too high; and (3) The microspheres tended to aggregate.

20

To investigate the sodium alginate/XAMA-7 crosslinking agent reaction, the reaction was conducted in aqueous solution without the emulsification system. These reactions were carried out in small glass vials; the sodium alginate and XAMA-7 crosslinking agent were mixed together in the vial. The time to gelation was taken as the time after mixing at which the gel no longer flowed when the sample vial was inverted.

25

Generally, the sodium alginate/XAMA-7 solution became progressively more viscous until, finally, it formed a near-solid gel that would not flow when the sample vial was inverted. According to the manufacturer, the XAMA-7 crosslinking agent reacts rapidly with carboxyl or hydroxyl groups at pH 7 but much more slowly at pH 11. Therefore, the time required for gelation in an aqueous solution of sodium alginate was measured at pH 7. The same pH was used in the experiments in which water or methanol or isopropanol was used in the absence of sodium alginate.

35

The results of these experiments are shown below in Table V.

TABLE V

The Effect of XAMA-7 Concentration at pH 7 on Crosslinking

5

## 2.0% Sodium Alginate in Water

Run No.	Water (g)	Sodium (g)	Alginate (%)	XAMA-7* (g)	Gelation Time (hr)
10 1	2.0	0.04	2.0	0.20	1.0
2	2.0	0.04	2.0	0.10	1.5
3	2.0	0.04	2.0	0.05	>24

## Water without Sodium Alginate

15

Run No.	Water (g)	Sodium (g)	Alginate (%)	XAMA-7* (g)	Gelation Time (hr)
4	2.0	----	---	0.20	2.7
5	2.0	----	---	0.10	48
20 6	2.0	----	---	0.05	>48

## Water with Methanol, No Sodium Alginate

Run No.	Water (g)	Methanol (g)	(%)	XAMA-7* (g)	Gelation Time (hr)
25 7	2.0	0.20	10.0	0.20	12
8	2.0	0.20	10.0	0.10	>12
9	2.0	0.20	10.0	0.05	>24

30

## Water with Isopropanol, No Sodium Alginate

Run No.	Water (g)	Isopropanol (g)	(%)	XAMA-7* (g)	Gelation Time (hr)
10	2.0	0.20	10.0	0.20	15
35 11	2.0	0.20	10.0	0.10	>15
12	2.0	0.20	10.0	0.05	no reaction

\* as a 5% solution

The results shown in Table V confirm that XAMA-7 is an active crosslinking agent. It reacted with the carboxyl and hydroxyl groups of the sodium alginate, and, in the absence of sodium alginate, it reacted with water or itself to give gelation times almost as short. It also reacted with the isopropanol used to stop the reaction or the methanol used to wash the microspheres.

The order of reactivity with the crosslinking agent is seen from the table to be sodium alginate (carboxyl groups), followed by water, methanol, and isopropanol. The side reaction of the crosslinking agent with water formed hydroxyl groups that could stabilize the microspheres. Also, the XAMA-7 crosslinking agent reacted with the isopropanol used to stop the reaction and the methanol used to wash the microspheres, and thus allowed the microspheres to be separated from the medium.

### Example 6

#### Effect of Concentration of Water-Soluble Polymer In Aqueous Solution Relative To That of Crosslinking Agent

The original ratio of the weight of sodium alginate/XAMA-7 crosslinking agent in the aqueous solution was 0.4. This ratio, it was discovered, is too low; indeed, the microspheres resulting from such a ratio comprised poly(XAMA-7) crosslinked with the sodium alginate polymer, rather than the opposite. Therefore, it was considered important to reduce the concentration of XAMA-7 crosslinking agent in the aqueous solution relative to that of the sodium alginate.

To each of four vials was added 2.0 g water, 1.0 g 2.0-5.0% sodium alginate solution, and 0.05 g XAMA-7 crosslinking agent as a 5% solution, at pH 7. The sodium alginate/XAMA-7 crosslinking agent ratio was increased from 0.4 to 1.0.

These reactions were investigated in aqueous solutions without the emulsifying system. The results of these experiments are given in Table VI below:

TABLE VI

The Effect of Sodium Alginate Concentration at pH 7

Run No.	Sodium Alginate Solution		XAMA-7*		Na Alg/ XAMA-7	Gelation Time
	(g)	(%)	(g)	Ratio	(hr)	
2-7-1	1.0		2.0	0.05	0.4	5.0
3-7-1	1.0		3.0	0.05	0.6	5.0
4-7-1	1.0		4.0	0.05	0.8	2.5
5-7-1	1.0		5.0	0.05	1.0	1.0

\* as a 5% solution

The properties of the crosslinked microsphere varied with the sodium alginate/XAMA-7 crosslinking agent ratio. This was shown by the behavior of the microspheres when they were dispersed in water and forced through the needle of a hypodermic syringe.

Increasing the sodium alginate concentration to 5.0%, as seen from Table VI decreased the gelation time from 5 hours to 1 hour. The higher viscosity of the 5% sodium alginate solution made it difficult, however, to mix it with the crosslinking agent solution. Nevertheless, the viscosity was still low enough for the experiments to be carried out in the laboratory.

**Example 7**Effect of Sodium Alginate Concentration At A Constant  
Concentration of Crosslinking Agent

This example shows the corresponding effect of increasing the sodium alginate concentration at a constant XAMA-7 crosslinking agent concentration at pH 8. These reactions, like those in Example 6, were investigated in aqueous solutions without the emulsifying system. The data is shown in Table VII.

**TABLE VII**  
**The Effect of Sodium Alginate Concentration at pH 8**

5	Run No.	Sodium Alginate Solution		XAMA-7*	Na Alg/ XAMA-7 Ratio	Gelation Time (hr)
		(g)	(%)			
	2-8-1	1.0	2.0	0.05	0.4	3.0
	3-8-1	1.0	3.0	0.05	0.6	3.0
10	4-8-1	1.0	4.0	0.05	0.8	1.5
	5-8-1	1.0	5.0	0.05	1.0	1.2

\* as a 5% solution

15           The results, as seen from Table VII, are similar to those at pH 7 (Example 6), except that the rate of gelation was slightly slower. Increasing the sodium alginate concentration from 2.0 % to 5.0% decreased the gelation time from 3 hours to 1.2 hours.

20

### Example 8

#### Effect of XAMA-7 Concentration and pH at Higher Concentrations Of Sodium Alginate

25           This example is to show the effect of the concentration of crosslinking agent and pH at higher concentrations of sodium alginate. These reactions, as in Example 6, were investigated without the emulsion system. The results are given in Table VIII.

TABLE VIII

Effect of XAMA-7 Concentration and pH  
at Higher Sodium Alginate Concentration  
6% Sodium Alginate in 2.0 g Water

5						
10	Run No.	pH	Sodium Alginate Solution (g)	XAMA-7 (g)	Na Alg/ XAMA-7 Ratio	Gelation Time (min)
	6-6-1	6.0	2.0	0.10	1.2	41*
	6-6-2	6.0	2.0	0.08	1.5	41*
	6-6-3	6.0	2.0	0.06	2.0	82*
	6-6-4	6.0	2.0	0.04	3.0	>240**
15						
	Run No.	pH	Sodium Alginate Solution (g)	XAMA-7 (g)	NaAlg/ XAMA-7	Gelation Time (min)
20						
						<u>7% Sodium Alginate in 2.0 g Water</u>
	7-7-1	7.0	2.0	0.10	1.2	11***
	7-7-2	7.0	2.0	0.08	1.5	11***
25	7-7-3	7.0	2.0	0.06	2.0	30****
	7-7-4	7.0	2.0	0.04	3.0	>30****
	7-8-1	8.0	2.0	0.10	1.2	30
	7-8-2	8.0	2.0	0.08	1.5	50
	7-8-3	8.0	2.0	0.06	2.0	180
30	7-8-4	8.0	2.0	0.04	3.0	>480
	7-9-1	9.0	2.0	0.10	1.2	30
	7-9-2	9.0	2.0	0.08	1.5	50
	7-9-3	9.0	2.0	0.06	2.0	180
	7-9-4	9.0	2.0	0.04	3.0	>480
35	7-10-1	10.0	2.0	0.10	1.2	45
	7-10-2	10.0	2.0	0.08	1.5	180
	7-10-3	10.0	2.0	0.06	2.0	---
	7-10-4	10.0	2.0	0.04	3.0	>480
	7-11-1	11.0	2.0	0.10	1.2	>48
40	7-11-2	11.0	2.0	0.08	1.5	>48
	7-11-3	11.0	2.0	0.06	2.0	>48
	7-11-4	11.0	2.0	0.04	3.0	---

\* turned cloudy in 5 min., opaque white in 30 min.

\*\* turned cloudy in 30 min., opaque white in 60 min.

\*\*\* turned cloudy in 3 min., opaque white in 6 min.

\*\*\*\* turned cloudy and opaque white in 12 min.

XAMA-7 added as a 5% solution

Table VIII shows the effect of XAMA-7 crosslinking agent concentrations and pH at 6.0 and 7.0% sodium alginate concentrations. The pH was varied from 6.0 to 11.0. Generally, the gelation times increased with increasing pH, e.g., from 11 minutes at pH 7 to more than 48 hours at pH 11. The gelation times at pH 6 were longer than those at pH 7.

In general, as seen from Table VIII, the gelation time increased with decreasing XAMA-7 concentration at a given pH, e.g., from 11 minutes at 0.10 g XAMA-7 crosslinking agent to more than 30 minutes for 0.04 g XAMA-7 crosslinking agent.

#### Example 9

##### Effect of Various Concentrations of XAMA-7 And Varying pH On Gelation Times For Sodium Alginate

The effect of the XAMA-7 crosslinking agent concentration at pH values ranging from 6.4 to 11.0 for a 6.0% sodium alginate concentration is shown in Table IX.



**TABLE IX**  
**Effect of XAMA-7 Concentration and pH**  
**at 6% Sodium Alginate Concentration**

6% Sodium Alginate in 2.0 g Water						
	Run No.	pH	Sodium Alginate Solution (g)	XAMA-7 (g)	Na Alg/ XAMA-7 Ratio	Gelation Time (min)
5						
10	6-6-1	6.0	2.0	0.10	1.2	41*
	6-6-2	6.0	2.0	0.08	1.5	41*
	6-6-3	6.0	2.0	0.06	2.0	82*
	6-6-4	6.0	2.0	0.04	3.0	>240**
15	6-6-1	6.4	2.0	0.10	1.2	96*
	6-8-1	8.0	2.0	0.10	1.2	96**
	6-9-1	9.0	2.0	0.10	1.2	125**
	6-10-1	10.0	2.0	0.10	1.2	---
	6-11-1	11.0	2.0	0.10	1.2	***
20	* turned cloudy in 6 min., opaque white at 25 min. ** turned cloudy in 9 min., opaque white in 25 min. *** very slow reaction					
	6-6-2	6.4	2.0	0.08	1.5	200*
25	6-8-2	8.0	2.0	0.08	1.5	200*
	6-9-2	9.0	2.0	0.08	1.5	200**
	6-10-2	10.0	2.0	0.08	1.5	>200***
	6-11-2	11.0	2.0	0.08	1.5	****
30	* turned opaque white in 42 min., formed immobile gel ** turned opaque white in 42 min., gel flowed slowly *** turned opaque white in 42 min., gel flowed easily **** clear liquid after 200 min.					

	Run No.	pH	Sodium Alginate Solution (g)	XAMA-7 (g)	Na Alg/ XAMA-7 Ratio	Gelation Time (min)
5	6-6-3	6.4	2.0	0.06	2.0	260*
	6-8-3	8.0	2.0	0.06	2.0	260*
	6-9-3	9.0	2.0	0.06	2.0	260**
	6-10-3	10.0	2.0	0.06	2.0	>260***
10	6-11-3	11.0	2.0	0.06	2.0	****
* turned opaque white in 45 min. ** turned opaque white in 45 min., gel flowed slowly *** gel flowed easily **** clear liquid after 260 min.						
15	6-6-4	6.4	2.0	0.04	3.0	300*
	6-8-4	8.0	2.0	0.04	3.0	300*
	6-9-4	9.0	2.0	0.04	3.0	300*
20	6-10-4	10.0	2.0	0.04	3.0	>300*
	6-11-4	11.0	2.0	0.04	3.0	**
* flowed when inverted ** remained a clear liquid XAMA-7 added as a 5% solution						
25						

The reactions investigated in Example 9 were made on the aqueous solutions without the emulsion system and were conducted as disclosed in Example 6.

At constant pH, the gelation time generally increased with decreasing XAMA-7 concentration, e.g., from about 100 minutes at 0.10 g XAMA-7 to about 200 minutes at 0.08 g and from about 260 minutes at 0.06 g to about 300 minutes at 0.04 g.

At a given XAMA-7 crosslinking agent concentration the gelation time generally increased with increasing pH, but the increase was not great. Nevertheless, none of the samples gelled within a reasonable time at pH 11. All samples gave the shortest gelation time at a pH 6.4 to 8.0.

These results show that the reaction between the sodium alginate and the XAMA-7 crosslinking agent can be controlled by the concentration of the reactants, as well as by pH.

### Example 10

#### Crosslinking of Water Soluble Polymers With Various Crosslinking Agents

Other crosslinking-in-solution experiments were carried out using different crosslinking agents and different water-soluble polymers. The results are shown below in Table X.

#### TABLE X

#### Crosslinking with Different Crosslinking Agents

Polymer	Glutaraldehyde	XAMA-7	Divinyl Sulfone	Borate
	pH 2	pH 2	pH 10	pH 8-9
PVA	0.10/0.10 precipitated	0.10/0.10 clear	not soluble clear	0.10/0.10 floaters
CS	0.20/0.20 clear	0.20/0.10 slight gel precipitated	0.20/0.20 clear	0.10/0.10 clear
		0.40/1.0 precipitated	0.40/1.0 precipitated	
		0.20/0.10 pH 7 opaque		
SA	0.20/0.20 pH4 opaque	0.10/0.10 pH 7 precipitated	0.20/0.20 clear	0.10/0.10 clear
		0.20/0.50 pH 7 highly gelled	0.40/0.10 highly gelled	

PVA = poly (vinyl alcohol)

CS = chondroitin sulfate

SA = sodium alginate

Borate = boric acid  $H_3BO_3$

In the above Table X, the first number refers to the weight of the polymer and the second number to the weight of the crosslinking agent in g, e.g., the "0.10/0.10" in the upper lefthand

column under Glutaraldehyde refers to a sample comprising 0.10 g poly(vinyl alcohol) i.e., PVA, and 0.10 g glutaraldehyde.

15 ml of the polymer solution containing the stated amount was mixed with the stated amount of crosslinking agent at room temperature. The pH was adjusted to the value set forth in the table with either sulfuric acid or sodium hydroxide. The formation of a visible gelled structure was taken as evidence that crosslinking had occurred.

The data shows that the glutaraldehyde (pH 2) crosslinked the poly(vinyl alcohol) and the sodium alginate (SA). The SA, however, crosslinked to a greater degree than the PVA. The glutaraldehyde failed to crosslink the chondroitin sulfate (CS). With XAMA-7, the CS gave a gel at the 0.20/0.10 ratio, a stiffer gel at the 0.40/0.10 ratio, and an opaque gel at the 0.20/0.10 ratio at pH 7. In the case of the sodium alginate, strong gels were given at pH 7 at both the 0.10/0.10 and 0.20/0.50 ratios.

The divinyl sulfone was insoluble in the aqueous poly(vinyl alcohol) at pH 10 and did not give a gel. With the chondroitin sulfate, no gel was given at the 0.20/0.20 ratio, but a gel was given at the 0.40/0.10 ratio. The sodium alginate gave no gel at the 0.20/0.20 ratio; however, a gel was formed at the 0.40/0.10 ratio.

The borate crosslinking agent at pH 8-9 gave structures that floated to the top of the sample in the case of the poly(vinyl alcohol). It did not crosslink the chondroitin sulfate or the sodium alginate.

### Example 11

#### Effect of Other Crosslinking Agents On The Crosslinking of Water-Soluble Polymer Particles

Other crosslinking agents tested include the carbodiimides and epoxide crosslinking agents. These investigations as in the previous example were made without the emulsion system. The results of the experiments are shown in Table XI below:

TABLE XI

Crosslinking with Different Crosslinking Agents

Polymer	<u>Carbodiimide, pH 4</u>		<u>Epoxide, pH 10</u>	
	<u>CHME-CDI</u>	<u>DMAPE-CDI</u>	<u>ECH</u>	<u>BDEP</u>
PVA	0.10/0.10 clear	0.10/0.10 clear	0.10/0.10 clear	0.10/0.10 clear
CS	0.18/0.18 clear		0.20/0.20 clear	0.20/0.20 clear
	0.36/0.18 clear		0.40/1.0 precipitated	0.20/0.20 clear
	0.18/0.18/0.05 precipitated			
	0.18/0.09/0.05 precipitated	0.18/0.09/0.06 precipitated		
SA	0.18/0.18 clear	0.18/0.09 clear	0.20/0.20 clear	0.20/0.20 clear
	0.18/0.09 clear	0.18/0.18 clear	0.40/0.05 clear	
	0.18/0.09/0.06 opaque, gelled	0.18/0.09/0.06 opaque, gelled		

CHME-CDI = 1-cyclohexyl-3- (2-morpholinoethyl) carbodiimide

DMAPE-CDI = 1-(3-dimethylaminopropyl) -3-ethylcarbodiimide

ECH = epichlorhydrin

BDEP = 1, 3-butadiene diepoxide

15 ml of the polymer solution containing the stated amounts of the polymers were mixed with the crosslinking agents at room temperature. The first number designates the weight in grams of the polymer; the second number designates the grams of crosslinking agent. Where a third number appears, this is the gram weight of 1, 5-diaminopentane dihydrochloride.

The pH was adjusted with sulfuric acid or sodium hydroxide to 4 for the carbodiimides and to 10 for epoxide crosslinking agents. The formation of a visible gelled structure was taken as evidence that crosslinking had occurred.

As can be seen from Table XI, the poly(vinyl alcohol) gave clear solutions with equal weights (0.10 g) of the 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (CHME-CDI), 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide (DMAPE-CDI),  
5 epichlorhydrin (ECH), and 1,3-butadiene diepoxide (BDEP), which indicates that the hydroxyl groups of the poly(vinyl alcohol) are not sufficiently active to react with any of these crosslinking agents.

The chondroitin sulfate gave clear solutions with equal weights (0.18 g) of the polymer and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide, and when the weight of the polymer  
10 was doubled to 0.36 g, which indicated that no significant crosslinking had occurred. When 0.05 g 1,5-diaminopentane dihydrochloride was added, along with 0.18/0.18, or 0.18/0.09, g of polymer/carbodiimide, the sample precipitated, indicating that  
15 crosslinking had occurred. Similar crosslinking was observed with the 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide. The 1,3-butadiene diepoxide gave no crosslinking at the ratios used. The epichlorhydrin gave cross-linking at the 0.40/1.0 ratio but not at the 0.20/0.20 ratio.

20 The sodium alginate did not crosslink with equal weights (0.18 g) of the polymer and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, nor when the weight of the crosslinking was cut in half (0.09 g); however, upon addition of 0.06 g 1,5-diaminopentane dihydrochloride, the sample was crosslinked to an opaque gel. The  
25 results were similar with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide. Equal weights (0.20 g) of epichlorhydrin and 1,3-butadiene diepoxide gave clear solutions, even when the amount of sodium alginate was increased to 0.40 g and that of the epichlorhydrin was reduced to 0.05 g, which indicated that no crosslinking had  
30 occurred.

## Example 12

### Preparation of Microspheres Of Water-Soluble Polymer By Inverse Emulsification Process Of Invention

5

Sodium alginate gel microspheres were prepared by using an inverse emulsification process. Table XII below gives the various recipes used in the inverse emulsification process.

Typically, 0.60 g of sodium alginate dissolved in 30.0 g of water was emulsified in 30.0 g toluene which contained 0.30 g SPAN 60, a water-in-oil emulsifier (1.0% by weight).

The samples were placed in 4 ounce glass bottles and shaken by hand to form the inverse emulsions of aqueous sodium alginate solution in toluene. The XAMA-7 crosslinking agent was next added to each bottle. The bottles were then capped and tumbled end-over-end for 24 hrs. at 25°C. During this time, the XAMA-7 crosslinking agent reacted with the sodium alginate and microspheres formed. After the reaction was completed, excess methanol was added to dehydrate the sodium alginate microspheres, which were then filtered out from the water phase and washed three times with methanol. The recovered and washed microspheres were then dried in an oven for 24 hours at 75°C.



**Table XII**  
**Recipes for the Preparation of Alginate Microspheres**

<u>Run No.</u>	<u>S-01</u>	<u>02</u>	<u>03</u>	<u>04</u>	<u>04B</u>	<u>06</u>	<u>07</u>	<u>08</u>
5 Sodium alginate (g)	0.60	0.60	0.60	0.60	0.60	0.00	0.90	1.2
10 Water (g)	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
10 Toluene (g)	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Span 60 (g)	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
15 XAMA-7 (g)	1.42	1.78	2.37	3.56	3.56	2.37	3.56	3.56
(ml)	3.00	1.20	1.50	2.00	3.00	3.00	2.00	3.00

Runs S-04 and S-04B, which used the highest concentration of crosslinking agent, gave spherical microspheres with a broad distribution of sizes. The microspheres were stable and withstood drying at 75°C overnight without distortion. The crosslinking reaction was judged to be essentially complete from the gravimetric material balance, e.g., in the case of Run S-04B, the original weight of sodium alginate plus that of the XAMA-7 crosslinking agent was 4.16 g, as compared to 4.02 g for the recovered weight of the microspheres. Samples of the microspheres were placed on microscope slides, washed with methanol to remove the SPAN 60, and examined by optical microscopy. All the microspheres were well-defined spheres, even after absorption of water. The microspheres of Run S-04 swelled and absorbed 63.6% water.

The XAMA-7 crosslinking agent concentration was increased at constant sodium alginate concentration in Runs S-01, S-02, S-03, and S-04. Generally, optical microscopy showed that both large and small microspheres were obtained. The larger microspheres appeared to be wrinkled in appearance, and only those of Run S-04 appeared to be spherical. These results were interpreted as the result of the reaction of the XAMA-7 crosslinking agent with the sodium alginate to give gelled microspheres.

The sodium alginate concentration was increased at constant (high) XAMA-7 crosslinking agent concentration in Run S-07 and Run S-08. These runs produced beads that were spheroidal rather than spherical in shape. The reason for the production of spheroidal beads is not actually known; however, possible reasons include non-uniform crosslinking of the original inverse emulsion droplets or partial coagulation of crosslinked beads.

In contrast, Run S-06, which contained the XAMA-7 crosslinking agent, but no sodium alginate, gave no beads. It gave, instead, an emulsion which disappeared within 30 minutes upon standing. Apparently, the sodium alginate, although present in only a small concentration, is necessary for the formation of a crosslinked network under these conditions. Condensation of the XAMA-7 crosslinking agent by itself does not, apparently, give a similar crosslinked structure.

### Example 13

#### Inverse Emulsification of Mixtures of Sodium Alginate and Methyl Cellulose To Obtain Crosslinked Microspheres and Size Distribution

Table XIII gives the recipes and results for inverse emulsifications of sodium alginate and METHOCEL K4M methylcellulose mixtures using the XAMA-7 crosslinking agent.

Typically, 50.0 g water containing 2.25 g dissolved sodium alginate and 0.25 g methyl cellulose (5% w/w sodium alginate/methyl cellulose) were dispersed in 75.0 g isooctane containing 1.5 g SPAN 85 in the one-half liter kettle C, earlier disclosed, and stirred for 10 minutes. Then, 5.0 g water containing 1.0 g TWEEN 85, a general purpose water soluble emulsifier (a polyoxyethylene derivative of fatty acid partial ester of sorbitol anhydride) was added to the dispersion and the dispersion was stirred for another 5 minutes. An equivalent amount of the XAMA-7 crosslinking agent or its aqueous solution was then added to the dispersion. The crosslinking agent was then allowed to react with the

dispersed polymer droplets for 180 minutes while being stirred, to crosslink the droplets. Then, 25 ml isopropanol was added to dehydrate and harden the microspheres formed. The stirring was continued for another 10 minutes, after which the mixture separated  
5 into a clear supernatant layer and an opaque, white sedimented microsphere layer. The filtration of the microspheres proved difficult; therefore, the supernatant layer was decanted, and the sedimented microspheres were washed twice by stirring overnight in a beaker with 200 ml isopropanol. Then, the microspheres were  
10 filtered easily on filter paper and dried overnight at room temperature.

Table XIII

Sodium Alginate/Methyl Cellulose Microspheres

5	<u>Run No.</u>	<u>WA-02</u>	<u>WA-03</u>	<u>WA-04</u>	<u>WA-05</u>	<u>WA-06</u>	<u>WA-07</u>
	Water Phase (g)						
	Sodium Alginate	2.25	2.25	2.25	2.25	2.25	2.25
	Methocel K4M	0.25	0.25	0.25	0.25	0.25	0.25
10	Water	50.0	50.0	50.0	50.0	50.0	50.0
	Oil Phase (g)						
	Isooctane	75.0	75.0	75.0	75.0	75.0	75.0
	Span 85	1.50	1.50	1.50	1.50	1.50	1.50
15	Emulsifier (g)						
	Tween 85	1.0	1.0	1.0	1.0	1.0	1.0
	Water	5.0	5.0	5.0	5.0	5.0	5.0
20	Crosslinking Agent (ml)						
	XAMA-7	11.3	10.0	9.0	9.0	6.5	11.3
	Water	50.0	50.0	---	---	---	---
25	Reaction Flask	C	C	C	C	C	C
	<u>Run No.</u>	<u>WA-02</u>	<u>WA-03</u>	<u>WA-04</u>	<u>WA-05</u>	<u>WA-06</u>	<u>WA-07</u>
	React. Time (min)	180	120	180	180	180	180
30	Microsphere Size (%)						
	a. >250 um	0.0	28.9	6.0	5.4	11.5	11.6
	b. 250-212 um	0.0	1.5	1.4	0.0	0.0	2.9
35	c. 212-150 um	5.0	2.9	3.0	1.8	2.6	7.2
	d. <150 um	93.8	66.6	90.5	92.5	86.0	75.0

These experiments show that crosslinked sodium alginate/Methocel K4M beads of diameters mostly smaller than 150 micrometers can be prepared reproducibly using isooctane as the continuous phase, SPAN 85 as the emulsifying agent, and XAMA-7 as the crosslinking agent.

## Example 14

Sodium Alginate Microspheres Prepared By Inverse  
Emulsification and Crosslinked By Calcium Ions

In this example, divalent calcium ions were used to crosslink the sodium alginate to obtain a comparison with the XAMA-7 crosslinking agent.

5           Calcium chloride was first used to crosslink sodium alginate about 20 years ago (M. Kiestan and C. Burke, *Biotechnol. Bioeng.* 19, 387-97 (1977)); a 2-4% aqueous sodium alginate solution was injected into a 2% aqueous calcium chloride solution to form large hydrophilic crosslinked sodium alginate beads. This process  
10 was not an emulsification in the usual sense, but instead produced microspheres by the crosslinking of sodium alginate by calcium ions at the interface between the aqueous sodium alginate and calcium chloride phases. In general, this method gave microcapsules with a broad distribution of sizes of 10-2000 micrometers diameter;  
15 however, it was difficult to prepare smaller microspheres of 0.1-200 micrometers diameter.

For this example, sodium alginate microspheres were prepared by inverse emulsification and crosslinked with calcium ions, according to the method used by Wan et al., *Proc. NUS-JPS Seminar on Recent Developments in Pharmaceuticals and Pharmaceutical Technology*, 1990, pp. 243-55; *ibid.*, *J. Microencapsulation* 9(3), 309-16 (1992), to encapsulate drugs. The disclosures in these publications are fully incorporated herein by reference.  
20

25           Table XIV below gives the recipes and results for these inverse emulsifications using calcium chloride as the crosslinking agent. Typically, 50.0 g aqueous solution containing 2.25 g dissolved sodium alginate and 0.25 g METHOCEL K4M (5% w/w sodium alginate/METHOCEL K4M) was dispersed in 75.0 g isooctane  
30 containing 1.5 g SPAN 85 in different reaction flasks and stirred at 1000 rpm for 10 minutes. Then, 5.0 g aqueous solution containing 1.0 g TWEEN 85 was added, and the dispersion was stirred for another 5 minutes. 25 g 8% w/w calcium chloride solution was added and allowed to react with the dispersed polymer droplets for 60  
35 minutes. The microspheres were then dehydrated using the method

developed by Ismail et al. (N.Ismail, M. S. Harris, and J. R. Nixon),  
J. Microencapsulation 1(1), 9-19 (1984), the entire disclosure of  
which is incorporated herein by reference, to dehydrate gelatin-  
acacia microcapsules, i.e, by stirring for 10 minutes in 25 ml  
5 isopropanol, filtering, washing twice by stirring overnight in a  
beaker with 200 ml isopropanol, filtering again, and drying at room  
temperature over night.

Table XIV

**Sodium Alginate Microspheres Crosslinked  
Using Calcium Chloride**

<u>Run No.</u>		<u>W-04</u>	<u>W-06</u>	<u>W-07</u>	<u>W-10</u>
10	Water phase (g)				
	Sodium alginate	4.50	9.00	9.00	2.25
	Methocel K4M	0.50	1.00	1.00	0.25
	Water	100.0	200.0	200.0	50.0
15	Oil phase (g)				
	Isooctane	150.0	300.0	300.0	75.0
	Span 85	3.0	6.0	6.0	1.5
20	Emulsifier (g)				
	Tween 85	2.0	4.0	4.0	1.0
	Water	10.0	20.0	20.0	5.0
25	Crosslinking Agent				
	8% aq. CaCl <sub>2</sub> (ml)	50.0	100.0	100.0	25.0
	Addition Time (min)	10	20	20	60
	Dehydrating Agent (ml)				
	Isopropanol	50.0	100.0	100.0	25.0
30	Reaction Flask	A	B	B	C
	Reaction Time (min)	35	45	45	90
35	Microsphere Size (%)				
	a. >250 um	30.5	6.2	11.2	5.60
	b. 250-212 um	3.3	0.72	2.2	0.0
	c. 212-150 um	29.0	1.7	5.4	0.0
	d. <150 um	34.0	91.4	81.4	91.7

The results of these experiments show that the first run in reaction flask A gave a broad distribution of microsphere sizes. Only about one-third of the particles were smaller than 150 micrometers in diameter.

The runs in reaction flasks B and C gave a narrower size distribution and a large proportion of microspheres smaller than 150 micrometers in diameter. Thus, most of the microspheres crosslinked with calcium chloride were smaller than 150 micrometers in diameter.

The size, shape, and surface characteristics of these microspheres were markedly affected by the stirring speed, rate of addition of calcium chloride solution, and shape of the reaction flask and mixer. A 20- or 60-minute addition time for the aqueous calcium chloride solution, generally, gave microspheres that were smaller than 150 micrometers in diameter.

The use of reaction flask A (round-bottom flask with a Teflon polytetrafluoroethylene half-moon stirrer blade) and a shorter addition time, gave fewer microspheres smaller than 150 micrometers in diameter and more microspheres of a diameter larger than 250 micrometers. Reaction flasks B and C both gave about 90% of the microspheres smaller than 150 micrometer in diameter, and for this reason are preferred over reaction flask A. Thus, the use of calcium chloride as the crosslinking agent resulted in the production of microspheres with a particle size smaller than 150 micrometers in diameter and a narrow size distribution, according to the stirring rate.

By way of comparison, most of the microspheres crosslinked with the XAMA-7 crosslinking agent and prepared in reaction flask C were also smaller than 150 micrometers in diameter. The size, shape, and surface characteristics of these microspheres were also markedly affected by the stirring speed, and the shape of the reaction flask and stirrer. Thus, it can be seen that the results for calcium ion as the crosslinking agent are roughly parallel to those of the XAMA-7 crosslinking agent. The addition of the calcium ion, however, is different from the addition of the XAMA-7 crosslinking agent which, for the best results, requires the variation of pH from pH 11 before and during emulsification to pH 7-8 after emulsification to achieve the desired particle size.



**Example 15****Capacity Of The Microspheres To Pass Through  
A 20- or 22-Gauge Syringe**

5

In this experiment, the capacity of the microspheres to pass through a 20- or 22-gauge syringe was determined. Generally, dried microspheres were dispersed in water and allowed to swell.

Then, the aqueous dispersion was loaded into a  
10 hypodermic syringe and was forced out through the needle. Aqueous dispersions with low sodium alginate/XAMA-7 ratios could not be extruded, whereas those with higher sodium alginate/XAMA-7 ratios gave dispersions that could be extruded into worm-like threads. It is generally desirable for the aqueous dispersion of microspheres to be  
15 extrudable through a syringe to form a long worm-like thread.

### Example 16

#### Size Distribution of Sodium Alginate Microspheres With Varying Concentration of Emulsifier and pH During Crosslinking Step

5

In this example, the sodium alginate/toluene/SPAN 60 emulsification system was used to show the effect of pH on the variation of the rate of crosslinking, whereby to control the emulsification. If the crosslinking is too rapid, the emulsification might not give a small enough droplet size before the viscosity of the aqueous phase increases to a value too great for dispersion.

10

Therefore, the emulsifications were carried out at a relatively high pH, such as pH 11; then, after the emulsion had been formed, the pH was lowered to 7. This approach allows ample time for the emulsification to be accomplished, yet provides rapid crosslinking once a desirable emulsion has been formed.

15

Table XV below gives the recipes for these experiments and the results obtained:

Table XV

Sodium Alginate Microspheres Using XAMA-7 and SPAN 60

5	Run No.	S-35	S-36	S-37	S-38	S-39*	S-40	S-42
	Water Phase (g)							
	Sodium alginate	7.0	7.0	7.0	7.0	7.0	7.0	7.0
	Water	100	100	100	100	100	100	100
10	Oil Phase (g)							
	Toluene	100	100	100	100	100	100	100
	SPAN 60	1.5	1.0	1.0	1.0	1.0	2.0	2.0
	pH (using NH <sub>4</sub> OH)	6.6	10.0	9.0	9.0	9.0	9.0	6.4
15	XAMA-7 (g)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
	pH (using aq. HCl)	---	7.0	---	---	---	---	---
20	Reaction flask	C	C	C	C	C	C	C
	Reaction time (hr)	5.0	4.0	3.0	4.5	4.5*	4.0	4.5
	Stirring rate (rpm)	1100	1100	1300	1000-1300	1000	1200	1300-1500
25	Dehydrating agent (ml)							
	Isopropanol	100	100	100	100	100	100	100
30	Microsphere size (%)							
	a. >250 $\mu$ m		14.4	27.4	0.83	4.89	1.87	4.28
	b. 212-250 $\mu$ m		7.4	10.1	2.00	1.99	1.74	12.6
	c. 150-212 $\mu$ m		21.7	22.7	14.5	8.69	36.1	15.0
	d. <150 $\mu$ m		56.5	39.9	81.4	84.5	60.3	66.8

\* 20 g 8% calcium chloride after 2 hr reaction

In Run S-36, 37% of the microspheres formed were larger than 212 micrometers and 40% were smaller than 150 micrometers. A higher percentage of microspheres smaller than 150 micrometers is desired; however, the microspheres of this sample were aggregated. In this case, the original pH of 10 was lowered by addition of hydrochloric acid. A white precipitate was formed, which was attributed to the precipitation of alginic acid by the hydrochloric acid.

In Run S-37, the pH of the 7.0% aqueous alginate solution was adjusted from 6.6 to 9.0 with ammonium hydroxide. Similarly, the pH of Runs S-38 and S-40 was adjusted to 9.0. In Run S-40, the toluene and SPAN 60 concentrations were increased to enhance the emulsification; however, this higher concentration of the oil phase also resulted in aggregation.

By way of comparison, at a lower concentration, i.e., 5% sodium alginate; at pH 7, a gelation time of one hour is possible. (See Run No. 5-7-1, Table VI ).

### Example 17

#### Preparation of Crosslinked Sodium Alginate Particles In Oil Phase Comprising Isooctane and Emulsifier Mixture

As the XAMA-7 crosslinking agent proved to be somewhat soluble in toluene, it was decided not to use the toluene/SPAN 60 system in further experiments. Therefore, another system using isooctane as the organic phase and a SPAN 85/TWEEN 85 emulsifier mixture was substituted. Table XVI gives the recipes and results for these experiments.

Table XVI

Sodium Alginate Microspheres Using  
XAMA-7/Isooctane/SPAN 85

Run No.	W-20	W-21	W-23	W-30
Water phase (g)				
10 Sodium alginate	2.25	2.25	4.50	4.50
METHOCEL K4M	0.25	0.25	0.50	0.50
Water	50	50	50	100
Oil phase (g)				
15 Isooctane	75	75	150	150
SPAN 85	1.5	1.5	3.0	3.0
Emulsifier (g)				
20 TWEEN 85	1.0	1.0	1.0	2.0
Water	5.0	7.0	7.0	1.0
Crosslinking agent (g)				
XAMA-7	---	1.5	5.0	---
25 8% CaCl <sub>2</sub> soln	25	10	---	50
Reaction flask	C	C	C	E
Reaction time (hr)	4.5	4.5	4.5	3.0
30 Stirring rate (rpm)	2000- 2500	1100- 1500	1000- 1500	1000
Dehydrating agent (ml)				
35 Isopropanol	25	25	70	100
Microsphere size (%)				
a. >250 $\mu$ m	0	10.3	---	0
b. 212-250 $\mu$ m	0	3.0	---	2.5
c. 150-212 $\mu$ m	0	9.4	---	5.0
40 d. <150 $\mu$ m	100	77.3	---	92.5

As seen from Table XVI, Run W-23 gives a typical result of these experiments. The droplet sizes were checked by optical microscopy at each step. Droplets of the desired size (smaller than 150 micrometers) were observed in all steps up to the addition of isopropanol, which was intended to stop the crosslinking reaction. This addition increased the viscosity to the point where it became

difficult to stir the reaction mixture after about 20 minutes, at which point the sample appeared to be coagulated. When diluted with water, the sample showed the presence of microspheres.

Table XVI shows the results of using a crosslinking agent comprising a mixture of calcium chloride/XAMA-7. Run W-21 demonstrates that such a mixed crosslinking agent results in 77% of the microspheres being smaller than 150 micrometers. However, many of the microspheres were nonspherical and elongated in shape.

In contrast, the higher stirring rate of Run W-20, along with the use of calcium chloride as the sole crosslinking agent, gave 100% microspheres smaller than 150 micrometers. Nevertheless, there was some aggregation.

Generally, as can be concluded from Runs W-20 and W-21, the size, shape, and size distribution of the microspheres were determined by the stirring rate and the design of the reaction flask and stirrer, as well as the dispersing agent. In these two Runs, as well as in Run W-23, the stirring rate varied throughout the experiments because the motor was unable to maintain a constant speed, when the load varied during the emulsification and crosslinking. This variation is reflected in the range of stirring rates shown in Table XVI for Runs W-20, W-21, and W-22.

This experiment was repeated using a constant-rate electric stirrer. The results are shown in Run W-30 in Table XVI. As can be seen from Run W-30, when the stirring rate remained constant, independent of the load, microspheres of crosslinked sodium alginate/METHOCEL K4M, predominately had a diameter of less than 150 micrometers when using calcium ion as the crosslinking agent, with isooctane as the continuous phase, and TWEEN 85 as the emulsifying agent.

### Example 18

#### Comparison of Microspheres of Sodium Alginate Crosslinked With Calcium Ion and XAMA-7

5

The main difference between the calcium ion and XAMA-7 cross-linking agent is that the calcium ions form ionic bonds, whereas the XAMA-7 crosslinking agent forms covalent bonds. Ionic bonds are influenced by the electrolyte in the surrounding medium and may dissolve if the composition of the medium changes sufficiently. In contrast, covalent bonds are unlikely to be affected by changes in the medium. Therefore, experiments were carried out to determine the relative stability of microspheres crosslinked with ionic and covalent bonds. Table XVII gives the results of these experiments.

#### Table XVII

##### Comparison of CaCl<sub>2</sub> Ionic and XAMA-7 Covalent Bonds

20

<u>Run No.</u>	<u>A</u>	<u>B</u>	<u>A-1</u>	<u>A-2</u>
Crosslinking Agent	CaCl <sub>2</sub>	XAMA-7	CaCl <sub>2</sub>	CaCl <sub>2</sub>
Preparation Run	W-30d	S-58d	W-30d	W-30d
Microspheres (g)	0.15	0.15	0.15	0.15
5% EDTA soln. (g)	5.0	5.0	5.0	---
2% Saline soln. (g)	5.0	5.0	---	5.0
18 hrs room temp.; no stirring	some disinte- gration	no disinte- gration		
additional 18 hrs. room temp.; with stirring	beads almost disinte- grated to viscous liquid	no disinte- gration	beads almost disinte- grated to viscous liquid	no disinte- gration

40

Preparation Runs W-30d and S-58d refer to the fraction of microspheres of Runs W-30 and S-58 that were smaller than 150 micrometers

45

Microspheres crosslinked with calcium chloride (Runs W-30d) and XAMA-7 (Run S-58d) were placed in 5% ethylenediamineacetic acid (EDTA) and 2% aqueous saline (NaCl) solution and allowed to stand for 18 hours without stirring. The microspheres crosslinked with calcium chloride had begun to disintegrate, whereas those crosslinked with XAMA-7 remained stable without distortion.

After an additional 18 hours at room temperature with stirring, the microspheres crosslinked with calcium chloride continued to disintegrate, whereas those crosslinked with XAMA-7 remained integral without distortion.

In separate experiments using the EDTA solution and saline solution alone, the microspheres crosslinked with calcium chloride disintegrated in the EDTA solution, but not in the saline solution. Thus, it is the complexing of the calcium ions by the EDTA that results in the breaking of the crosslinks and the dissolution of the microspheres. The saline solution had no such effect.

In contrast, the microspheres crosslinked with XAMA-7 showed no disintegration in EDTA solution or saline solution under the same conditions. These results demonstrate that the covalent bonds resulting from the XAMA-7 crosslinking agent were more stable than the ionic bonds resulting from the calcium chloride crosslinking.

25

### Example 19

#### Preparation of Aqueous Dispersions of Protein Particles Crosslinked With Glutaraldehyde

30

In this example, aqueous dispersions of crosslinked particles of bovine serum albumin were prepared.

1.0 g bovine serum albumin was dissolved in 0.1 M sodium acetate buffer (pH 7.8) and 0.5 g glutaraldehyde was added to determine if the bovine serum would be crosslinked by the

35



crosslinking agent; the crosslinking action occurred immediately upon addition of the glutaraldehyde.

Then, 1.0 g bovine serum albumin was dissolved in 8 g buffer solution, and 2.0 g 25% glutaraldehyde was added after  
5 emulsification in 20.0 g o-xylene containing 5% PLURONIC L92, a commercially available non-ionic propylene oxide/ethylene oxide block copolymer emulsifier from BASF, having an HLB of 1.0-7.0. The crosslinking agent was allowed to react with the emulsified bovine serum albumin droplets for four hours at room temperature.  
10 The emulsion was then inverted in 400 ml water containing 0.5% AEROSOL A-102 wetting agent (disodium ethoxylated alcohol half-ester of sulfosuccinic acid) available from American Cyanamid Co.

### Example 20

15

#### Preparation of Water-In-Oil Dispersion of Crosslinked Particles of Bovine Serum Albumin

In this example, 3.0 g bovine serum albumin in 15.0 g  
20 buffer solution (sodium acetate pH 7.8) was emulsified in 60 g o-xylene containing 5% TETRONIC 1102, a commercially available (BASF ) water-in-oil emulsifier, HLB 6.5, which comprises ethylenediamine tetrasubstituted with propylene oxide/ethylene oxide block copolymers with 20 moles propylene oxide and 10 moles  
25 ethylene oxide. This emulsion was then ultrasonified with a Branson Ultrasonifier (50% duty cycle; setting 7; 1 min.). Afterwards, 6.0 g 25% aqueous glutaraldehyde solution was added to crosslink the bovine serum albumin droplets in the water-in-oil-dispersion.

The result was a dispersion of submicroscopic  
30 crosslinked bovine serum albumin particles dispersed in the continuous o-xylene phase.

### Example 21

#### Preparation of Aqueous Dispersion of Crosslinked Particles of Bovine Serum Albumin

5

6 g bovine serum albumin in 15 g buffer (sodium acetate pH 7.8) was emulsified in 60 g o-xylene containing PLURONIC L92 emulsifier and crosslinked with 8 g 25% aqueous glutaraldehyde solution added after emulsification. This water-in-oil dispersion of crosslinked bovine serum albumin particles was then inverted in 400 ml water containing 0.5% TRITON X-405, a commercially available octylphenoxy polyethoxy ethanol surfactant (Union Carbide; HLB 17.9)

15

The result of the inversion was a dispersion of crosslinked bovine serum albumin particles dispersed in water. The o-xylene continuous phase and water phase were immiscible and were easily separated from one another. Afterwards, the colloidal stability of the particle dispersion in water was tested, as described below.

20

The aqueous dispersion of crosslinked particles of bovine serum albumin was washed with buffer and then centrifuged twice, in a conventional Sorvall laboratory centrifuge. Afterwards the aqueous dispersion was tested for stability in aqueous sodium chloride solution. The dispersion showed good stability in 0.15 M sodium chloride, formed only a little coagulum in 0.30 M solution, more coagulum in 0.60 M solution and flocculated in 0.90 M solution. These results indicate that the bovine serum albumin dispersion is a lyophilic colloid rather than a lyophobic colloid, which generally have critical coagulation concentrations of about 0.1 M for monovalent cations. Transmission microscopy showed that the crosslinked particle size is from about 5 to about 8 micrometers.

30

## Example 22

### Effect of Crosslinking Concentration In The Formation of Aqueous Dispersions of Bovine Serum Albumin Particles

5

The effect of crosslinking concentration in the preparation of aqueous dispersions of crosslinked bovine serum albumin particles was determined by varying the glutaraldehyde concentration.

10           10 g 20% bovine serum albumin was emulsified in 30 g o-xylene containing approximately 5% TETRONIC 1102. The emulsion was ultrasonified (setting 5; 90 sec), and the glutaraldehyde was added to a concentration of 5, 10, 15, or 20% weight/weight, based on the bovine serum albumin. The crosslinking agent in each  
15 of the emulsions was then allowed to react for 12 hours at room temperature. Then, the emulsions were each inverted in 200 ml water containing 1% TRITON X-405. The o-xylene was removed by distillation of the o-xylene/water azeotrope. The dispersions were then each cleaned by serum replacement with water containing 50  
20 ppm formaldehyde (to prevent bacterial growth) and examined by transmission electron microscopy. In serum replacement, the dispersion or latex is confined in a cell with a semipermeable membrane, and pure water (or a solution) is pumped through the cell to literally replace the serum with the water (or solution). The net  
25 effect is to remove any dissolved material in the aqueous phase, as well as any surfactant adsorbed on the particles. Nuclepore membranes, which have a remarkably uniform submicroscopic pore size are used. The resolution of separation is good enough to remove the adsorbed and solute surfactant quantitatively. Each of the  
30 dispersions comprised discrete polymer particles dispersed in water.

### Example 23

#### Preparation of Aqueous Dispersions of Crosslinked Particles of Human Gamma-Globulin

5

In this example, three experiments were conducted to obtain aqueous dispersions of crosslinked human gamma-globulin particles. First, the rate of crosslinking of human gamma-globulin was determined. 1 g human gamma-globulin was dissolved in 10.5 g water, and this solution was then mixed with 6% and 12% aqueous glutaraldehyde solutions by weight, based on the weight of gamma globulin. Crosslinking occurred within 30 seconds after the addition of the crosslinking agent.

Then, 1 g human gamma-globulin in 10 g water was emulsified in 30 g o-xylene containing 1.5 g TETRONIC 1102. This water-in-oil dispersion of droplets of human gamma-globulin was then mixed with 0.24 g of 25% glutaraldehyde solution. This dispersion, when inverted in 400 ml water containing 0.5% AEROSOL A-102, coagulated.

7.8 g 10% aqueous human gamma-globulin solution was emulsified in 15 g o-xylene containing 0.75 g TETRONIC 1102. This emulsion was then ultrasonified as before and 0.17 g of 25% glutaraldehyde was added. The emulsion was then inverted in 150 g water containing 0.75 g AEROSOL A-102.

20 g 10% human gamma-globulin aqueous solution was emulsified in 60 g o-xylene containing 3 g TETRONIC 1102, after which the emulsion was ultrasonified, as earlier disclosed. During this time 0.8 g of glutaraldehyde (25% aqueous solution) was added to crosslink the human gamma-globulin droplets formed by the emulsion. The emulsion was then inverted in 400 ml water containing 2 g AEROSOL A-102.

Each of the above three dispersions, when examined under electron microscopy or optical microscopy, comprised crosslinked human gamma-globulin polymer particles in an aqueous dispersion.

## Example 24

### Preparation of Crosslinked Particles of Human Gamma-Globulin In Aqueous Dispersion

5

In this experiment, 1 g human gamma-globulin was dissolved in 9 g water and then emulsified in 30 g o-xylene containing 1.5 g TETRONIC 1102. The emulsion was ultrasorified, as before described, i.e., setting 5; 1 min. Then, 0.6 g of 15% glutaraldehyde solution was added, and the sample was tumbled end-over-end for 12 hours at room temperature in a capped bottle. The emulsion was then inverted in 300 ml water containing 3 g TRITON X-405. The o-xylene was then stripped at temperatures below 20°C to prevent aggregation of the particles. The aqueous dispersion, when cleaned by serum replacement in conventional manner, contained aggregates of the human gamma-globulin particles, which was attributed to the low pH. In any event, after serum replacement, the particles were seen to be aggregated.

In view of the above, the experiment was repeated except that the concentration of glutaraldehyde was doubled and the pH was adjusted to 7 by addition of sodium bicarbonate buffer. The dispersion was then inverted and cleaned by serum replacement with 0.1 M sodium acetate buffer (pH 7.8) at temperatures below 35°C to prevent aggregation. The result was that the particles were not aggregated.

Human gamma-globulin (2.5%) aqueous dispersions were also prepared in borate buffer (pH 8.-8.7). Only part of the human gamma-globulin was soluble in the borate buffer at 2.5% concentration.

1 g human gamma-globulin in 9 g buffer (pH 8.4) was emulsified in 30 g o-xylene containing 1.5 g TETRONIC 1102. The emulsion was ultrasorified as before (setting 3:30 sec), and 1.0 g of 25% aqueous glutaraldehyde added. The crosslinking reaction was carried out for 19 hours at room temperature. The dispersion was then inverted in an excess of 1% TRITON X-405 solution and agitated for 3 hours. The o-xylene was then stripped under vacuum

and the dispersion was cleaned by serum replacement with borate buffer (pH 8.4). As will be appreciated by those skilled in the art, significant dilution is necessary for distillation of the o-xylene/water azeotrope from the dispersion.

5           The human gamma-globulin particles were also crosslinked by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-hydrochloride (ECDI). 1.0 g 2% human gamma-globulin aqueous solution was mixed with 0.03, 0.10, 0.50, and 1.0% ECDI crosslinking agent, and with glutaraldehyde (25% aqueous  
10 solution) for comparison. A visible precipitate formed in each case, although the precipitate was somewhat difficult to see in the low concentration.

          Other TETRONIC water-in-oil emulsifiers, commercially available from BASF, such as TETRONIC 90R4 (HLB  
15 7.1), 110R2 (HLB 3.5), and 150R4 (HLB 5.4), were also tried. These emulsifiers all have low HLB values and are believed to be block copolymers of propylene oxide/ethylene oxide of varying proportions, similar in structure to TETRONIC 1102. These emulsifiers, however, were found not to be as effective in these  
20 emulsions as TETRONIC 1102. The TETRONIC 90R4 was found, moreover, not to be soluble in o-xylene.

### Example 25

Preparation of Aqueous Dispersion of Human Gamma-Globulin  
Particles Using Phosphate Buffer Saline  
As Medium for Emulsification

Phosphate buffered saline (pH 6.95) was tried as the medium for the emulsification of human gamma-globulin. In this case, 2.5% human gamma-globulin was soluble in this buffer. 10 g of 2.5% human gamma-globulin in phosphate buffered saline was emulsified in 30 g o-xylene containing 5% TETRONIC 1102. The emulsion was ultrasonified as before and mixed with 0.0125 g of ECDI crosslinking agent. The mixture was agitated for 4 days; after which the emulsion was inverted in 400 g phosphate buffer saline containing 0.5% TRITON X-405 and the o-xylene stripped, to give an aqueous dispersion of crosslinked human gamma-globulin particles.

### Example 26

Preparation of Aqueous Dispersions of Human Gamma-Globulin  
Using Various Concentrations of Human Gamma-Globulin and  
Crosslinking Agent ECDI

Other latexes were prepared using 10 g 3.5 % human gamma-globulin in 30 g o-xylene containing 5% TETRONIC 1102 and 0.035 g ECDI; 3 g 10% human gamma-globulin with 0.035 g ECDI in 10 g o-xylene containing 5% TETRONIC 1102; and 3 g 10% human gamma-globulin in 10 g o-xylene containing 5% TETRONIC 1102 with 0.06 g ECDI.

All of these latexes gave aqueous dispersions of crosslinked human gamma-globulin particles.

### Example 27

5

#### Distribution of Particle Sizes of Crosslinked Microspheres Prepared From Various Water-Soluble Polymers

10 In this example, crosslinked hydrophilic particles were prepared from hydroxyethyl cellulose, poly(vinyl alcohol), chondroitin sulfate, and other water-soluble polymers. Table XVIII gives the results for hydroxyethyl cellulose.



Table XVIII

Crosslinking of Hydroxyethyl Cellulose with XAMA-7

5	Run No.	HOC-01	-02	-03	-04
	Water Phase (g)				
	HOC	7.0	7.0	7.0	7.0
	Water 100	100		100	100
10	pH 7-8	7-8	7-8	10-11	
	Oil phase (g)				
	Span 60	1.0	1.0	1.0	1.0
	Toluene 100	100	100	100	
15	XAMA-7 (g)	4.0	4.0	4.0	4.0
	Dehydrating agent (ml)				
20	Methanol 100	---	---	---	
	Isopropanol	---	100	100	100
	Reaction flask	C	C	C	C
25	Stirring rate (rpm)	1000	1000	1000	1000
	Reaction time (hr)	19	14	18	22
30	Microspheres (%)				
	a. >250 um	---	7.5	19.5	---
	b. 212-250 um	---	2.3	5.9	---
	c. 150-212 um	---	1.8	29.7	---
	d. <150 um	---	88.7	45.0	---
35	HOC - hydroxyethyl cellulose				

The hydroxyethyl cellulose was dissolved in the water,  
 and the pH of the solution was adjusted to 7-8. The SPAN 60 was  
 40 dissolved in the toluene, and the solution was used as the continuous  
 phase for the emulsification of the aqueous hydroxyethyl cellulose  
 solution. The emulsification was carried out until the emulsion  
 droplets were judged satisfactory, i.e. the droplets were seen to be  
 discrete and spherical, and not irregular in shape, with a  
 45 preponderance of the sizes smaller than 150 micrometers diameter,

as monitored by optical microscopy. At pH 7, the crosslinking reaction (XAMA-7) occurred rapidly at room temperature.

In Run H0C-01, the beads aggregated upon addition of the methanol, to dehydrate the crosslinked particles formed.  
5 Therefore, in the remaining experiments shown in Table XVIII, isopropanol was used as the dehydrating agent. This resulted in the formation of microspheres of the stated size ranges. Generally, however, the microspheres were not as perfect as those made from sodium alginate.

10

### Example 28

Experiments Showing The Effect of Heat With And Without A Catalyst Combined With The Crosslinking Agent On The  
15 Preparation of Crosslinked Water-Soluble Particles

In these experiments, other water-soluble polymers were investigated, along with determining the effect of heat and a catalyst with the crosslinking agent. The results are shown in Table XIX  
20 below. In general, the procedure was: a) the dissolution of the water-soluble polymer in water, along with the crosslinking agent; b) emulsification of this solution in a continuous oil phase containing a water-in-oil emulsifier; c) homogenization of the emulsion by stirring, ultrasonification, or homogenization; d). heating of the  
25 emulsion to effect crosslinking; e). and inversion of the emulsion into water to transfer the crosslinked particles to the aqueous phase.

Table XIX

Crosslinking of Water-Soluble Polymers

5	<u>Run. No.</u>	<u>101</u>	<u>102</u>	<u>103</u>	<u>105</u>	<u>106</u>	<u>107</u>	<u>108</u>	<u>109</u>
	Polymer	PVP	PVP	PVP	PVA	PVA	MC	PVA	PVA
	Weight (g)	1.0	1.0	2.0	2.3	6.0	3.0	6.0	3.0
10	Water (g)	19.0	19.0	18.0	20.2	27.0	36.0	25.5	33.0
	Glutaraldehyde ---		---	---	2.5	7.0	3.0	7.0	3.8
15	o-Xylene (g)	20.0	20.0	40.0	22.5	40.0	40.0	43.2	18.0
	Span 60 (g)	1.0	0.2	1.0	---	---	---	---	---
	Tetronic 1102	---	---	---	2.5	4.0	4.0	4.8	2.0
20	p-Toluene (g)								
	sulfonic acid	1.0*	1.0*	2.0*	---	1.0	1.0	0.5	0.3
25	Inverting emulsifiers	---	---	---	---	---	---	X-405	X-405

\* ammonium persulfate

X-405 - Triton X-405

PVP - poly (N-vinyl pyrrolidone); MW 360,000

30 PVA - Vinol 125 poly(vinyl alcohol); MW 70,000; 99.6% hydrolyzed

MC - Methocel K4M hydroxypropylmethyl cellulose

Run No.	110	111	112	113	114	115	116	117
Polymer	MC	PVA	PVA	MC	PVA	PVA	PVA	PVA
5 Weight (g)	2.0	5.0	5.0	2.0	2.0	0.8	3.0	6.0
Water (g)	31.5	15.0	15.0	29.5	21.0	8.7	29.3	57.0
10 Glutaraldehyde	4.5	1.0	1.0	2.5	0.5	0.5	0.5	1.0
o-Xylene (g)	18.0	97.2	45.0	153.0	52.3	19.0	57.0	97.0
Tetronic 1102	2.0	10.8	5.0	17.0	0.25	---	3.0**	---
15 Pluronic L92	---	---	---	---	2.5	---	---	3.0
Aerosol OT	---	---	---	---	---	1.0*	---	---
20 p-Toluene sulfonic acid	0.25	0.25	0.8	1.0	0.1	0.05	0.2	0.3
Inverting	X-405	SLS	SLS	SLS	---	---	X-305	X-305
25 emulsifiers	SLS	---	---	---	---	---	A-102	A-102
	---	---	---	---	---	---	SLS	-----

\* also Tetronic 702 and 1102; Pluronic L92

\*\* also Tetronic 702, 70R2, 50R4; Pluronic L92; Span 60; AerosolOT

30 PVA - Vinol 125 poly(vinyl alcohol); MW 70,000; 99.6% hydrolyzed

MC - Methocel K4M hydroxypropylmethyl cellulose

The ammonium persulfate in the presence of p-toluene sulfonic acid was used as a crosslinking agent to crosslink the PVP.

35 In Run 101, a 5% aqueous solution of poly(N-vinyl pyrrolidone), containing the stated amount of ammonium persulfate as the crosslinking agent was emulsified in o-xylene. The emulsion was ultrasonified (Branson Ultrasonifier; 50% duty cycle setting; room temperature) and then heated for 30 minutes at 80°C. Upon  
40 inversion in water containing TRITON X-305 (octylphenoxypolyethoxy ethanol, HLB 17.3, Union Carbide), the presence of particles was demonstrated by optical microscopy and scanning electron microscopy, according to usual techniques.

Run 102 by comparison used a 5% aqueous solution of  
45 the PVP and ammonium persulfate as the crosslinking agent with, however, a 1% SPAN 60 solution being used rather than the 5% of Run 101. The emulsion was ultrasonified (50% duty cycle; setting 3;

1min.) and heated for 30 min. at 80°C. The result was a very viscous emulsion that could not be inverted. The viscous emulsion was believed to be due to the lower concentration of SPAN 60.

Run 103 used a larger batch size and an intermediate  
5 concentration of SPAN 60, i.e., 2.5%. The emulsion was hand-homogenized (two cycles) to reduce the droplet size to about 4 micrometers, stirred at 236 rpm using a conventional laboratory stirrer and heated at 80°C for 30 minutes. When inverted in water, the emulsion separated into two layers, the lower of which appeared  
10 to be a gel of the 4 micrometer diameter crosslinked particles.

In Run 105, an aqueous solution of poly(vinyl alcohol) was prepared and emulsified in o-xylene containing TETRONIC 1102 emulsifier. This emulsion was mixed with the glutaraldehyde crosslinking agent. This system failed to react after 1.5 hours at  
15 70°C. When 1.0 g p-toluene sulfonic acid in 11.0 g water was added, however, to 6.0 g PVA dissolved in 28 g of 28% glutaraldehyde, a rapid reaction occurred when the mixture was heated to 40°C. Similar experiments using methylcellulose (instead of the PVA) with glutaraldehyde crosslinking agent, and p-toluene sulfonic acid and  
20 hydrochloric acid catalysts, gave a brown color but no discernible particles. Run 106 used PVA as the water-soluble polymer and glutaraldehyde as the crosslinking agent, with p-toluene sulfonic acid as a catalyst.

Run 107 used methyl cellulose (Dow METHOCEL  
25 K4M) as the water-soluble polymer and glutaraldehyde as the crosslinking agent, catalyzed with p-toluene sulfonic acid. The reaction was conducted at room temperature. Water was removed from the emulsion by distillation of the o-xylene/water azeotrope, and an additional 61 g o-xylene was added to the emulsion. This  
30 resulted in some flocculation. The emulsion was then inverted in water to form a dispersion of crosslinked methyl cellulose particles in water.

Run 108 used PVA as the water-soluble polymer and the same crosslinking agent and catalyst as in Run 107, the later being  
35 added to the aqueous solution prior to emulsification. The PVA

crosslinked within 4 minutes after addition of the catalyst. The final particles were soft, and stuck to each other and the metal sphere for grinding. The emulsion was inverted in 500 ml aqueous TRITON X-405, and 26 ml o-xylene was removed by distillation. Some  
5 flocculation of the particles occurred.

Run 109 used PVA and the same crosslinking agent and catalyst as in Run 108. The glutaraldehyde and the p-toluene sulfonic acid were mixed together, and the solution was emulsified immediately thereafter. Therefore, the crosslinking reaction was  
10 somewhat competitive with the emulsification, and some crosslinking would have been expected along with the emulsification. A magnetic stirring rod was used to mix the sample. Although a magnetic stirring rod was effective in making the emulsion, those skilled in the art will readily appreciate that such is not capable of fine variation.  
15 The crosslinking reaction took place within 10 minutes. An additional 40 g o-xylene was added, while the o-xylene/water azeotrope was being distilled off the emulsion. The final emulsion was inverted in 300 ml 10% aqueous TRITON X-405 emulsifier and 500 ml 1% aqueous sodium lauryl sulfate, a high-HLB emulsifier,  
20 used to invert the water-in-oil emulsion. The emulsion inverted but with some flocculation of the crosslinked particles.

In Run 110, methyl cellulose was used as the water-soluble polymer. The crosslinking agent was glutaraldehyde and p-toluene sulfonic acid was used as a catalyst, these being added to the  
25 aqueous solution prior to emulsification. The polymer solution was emulsified in the oil phase immediately after addition of the glutaraldehyde and p-toluene sulfonic acid. This very viscous, i.e., hard to pour, solution was dispersed in the o-xylene using a conventional hand-homogenizer.

30 Crosslinking was judged complete within 2 hours at room temperature after addition of the catalyst and crosslinking agent. The emulsion was inverted in an aqueous solution comprising 200 ml 15% aqueous TRITON X-405 solution and 200 ml 1% aqueous sodium lauryl sulfate solution. Flocculation occurred during

this inversion. No particles were seen by scanning electron microscopy.

Run 111 used PVA as the water-soluble polymer and the same crosslinking agent and catalyst as in the previous run. The crosslinking agent and catalyst were added before the emulsification and mixed by ultrasonification. When 30 ml of the water-in-oil emulsion were inverted in 270 ml 1% aqueous sodium lauryl sulfate solution, and the o-xylene/water azeotrope was removed by distillation, the resulting water-in-water emulsion showed no particles by scanning electron microscopy. By comparison, when 20 g of the water-in-oil emulsion was stirred with 1.0 g p-toluene sulfonic acid for 30 hours at room temperature no crosslinking occurred, this being shown by the lack of particle formation. Nevertheless, when the water-in-oil emulsion was heated to 50°C crosslinking occurred, as was confirmed by the presence of particles.

Run 112 used 20 g 20% PVA and 16 g 25% glutaraldehyde with 4 g p-toluene sulfonic acid catalyst emulsified in 50 g o-xylene containing about 5 g TETRONIC 1102. This emulsion was heated at 40°C. When the catalyst was added before the emulsification, the water-soluble polymer solution became very viscous and could not be stirred. Nevertheless, when the catalyst was added after the emulsification, following the emulsion being rotated end-over-end in a capped bottle for 30 hours at room temperature, crosslinking occurred, as evidenced by the formation of beads. When the emulsion was inverted in 800 ml 1% aqueous sodium lauryl sulfate solution, it formed a precipitate, which was redispersed in the water with a hand-homogenizer.

Run 113 used 10 g 10% methyl cellulose solution and the stated amounts of crosslinking agent and catalyst as the water-soluble polymer solution. This solution was emulsified in an oil phase comprising o-xylene and 10% TETRONIC 1102 emulsifier. The emulsion was stripped according to usual techniques at 40°C to remove the o-xylene/water azeotrope (25 g water and 55 g of the o-xylene). 30 g of this emulsion, inverted in 300 ml of a 1% sodium lauryl sulfate solution showed little sign of reaction. Another 100 g

sample of the emulsion was inverted in 900 ml 0.5% sodium lauryl sulfate with similar results. This run was repeated using 20 g 10% methyl cellulose, 10 g 25% glutaraldehyde and 5 g 20% p-toluene sulfonic acid catalyst solution emulsified in 170 g o-xylene containing  
5 10% TETRONIC 1102. The emulsion was homogenized twice in a conventional hand-homogenizer. The emulsion was still stable after 20 ml water was stripped. The crosslinking reaction was carried out by tumbling the emulsion in a capped bottle end-over-end for 24 hours at 40°C. The polymer precipitated, and the solution became  
10 clear, this occurring before inversion as in the previous part of this Run 113.

In Run 114, 20 g 10% poly(vinyl alcohol) (VINOL 124; MW 90M) solution, 2 g 25% glutaraldehyde, and 0.5 g 20% p-toluene sulfonic acid solution was emulsified in 50 g o-xylene  
15 containing 5% PLURONIC L92. Some coagulum was formed after 12 ml water and 28 ml o-xylene was stripped. The emulsification was also carried out in 50 g o-xylene containing 5% TETRONIC 1102. This emulsion flocculated after 18 g water was stripped and the emulsion was shaken in a conventional Red Devil Paint Shaker.

20 Run 115 used an aqueous water-soluble polymer solution (8 g 10% PVA), 2 g 25% glutaraldehyde solution, and 0.2 g 25% p-toluene sulfonic acid solution emulsified in 20 g o-xylene containing 5% AEROSOL OT (the dioctyl ester of sodium sulfosuccinic acid), commercially available from American Cyanamid. In this Run, the  
25 emulsion was formed and then the catalyst was added. The polymer emulsion droplets were crosslinked within 30 minutes after the catalyst was added to the emulsion. The emulsion flocculated, however, after being shaken in a Red Devil Paint Shaker. Other emulsifications, carried out in o-xylene containing 5% TETRONIC  
30 702 ( BASF; HLB 1.0-7.0, believed to be a propylene oxide/ethylene oxide block copolymer ) with the catalyst added after emulsification, also gave flocculation. TETRONIC 1102 gave a stable emulsion with only a little coagulum, which was inverted in 500 ml 0.5% sodium lauryl sulfate solution. PLURONIC L92 gave a stable emulsion with  
35 only a little coagulum, which was inverted in 300 ml 0.2% sodium



lauryl sulfate solution, 300 ml 0.2% TRITON X-305 solution, or 200 ml 0.4% AEROSOL A-102 solution, without flocculation. On the other hand, AEROSOL OT gave a flocculation to form a mass of polymer.

- 5 In Run 116, 30 g 10% PVA aqueous solution, 2 g 25% glutaraldehyde aqueous solution, and 1 g 20% p-toluene sulfonic acid aqueous solution were emulsified in o-xylene (60 g) containing 6.0% TETRONIC 1102. The water was stripped from the emulsion, prior to the catalyst being added. Crosslinking of the polymer emulsion
- 10 droplets was carried out by tumbling the emulsion in a capped bottle end-over-end for 12 hours at room temperature. The emulsion, when inverted in 800 ml 0.2% TRITON X-305 or AEROSOL A-102 solution, flocculated, but was redispersed by ultrasonification (Branson Ultrasonifier; room temperature). Other emulsifiers were
- 15 compared using 8 g 10% PVA aqueous solution, 2 g 25% glutaraldehyde aqueous solution, and 0.2g (25%) p-toluene sulfonic acid aqueous solution, these solutions being emulsified in 20 g o-xylene containing 5% emulsifier. In each case, the first step was carrying out the emulsification using the hand-homogenizer.
- 20 TETRONIC 1102 gave a very stable emulsion; TETRONIC 702 gave an emulsion that separated upon cessation of the agitation. PLURONIC L92 gave a very stable emulsion, as did AEROSOL OT. SPAN 60, TETRONIC 70R2 and TETRONIC 50R4 each gave emulsions that separated on cessation of the agitation.

- 25 The second step in these experiments was the crosslinking of the polymer emulsion droplets in the emulsion; p-toluene sulfonic acid was added after emulsification, and the capped bottles in which the emulsions were contained were tumbled end-over-end at room temperature for 12 hours. The TETRONIC 1102
- 30 emulsion remained stable; the TETRONIC 702 emulsion crosslinked to form a mass of the water-soluble polymer; the PLURONIC L92 emulsion formed a small amount of coagulum, but otherwise gave a stable emulsion. The AEROSOL OT emulsion flocculated to a mass of polymer.

The third step in these experiments was the inversion of the emulsion in water containing 0.5% TRITON X-305, AEROSOL A-102, or sodium lauryl sulfate (all being high-HLB emulsifiers). The TRITON X-305 and AEROSOL A-102 each gave stable  
5 emulsions from which the o-xylene was easily distilled; the sodium lauryl sulfate formed a little coagulum, and the o-xylene was distilled only with difficulty. The AEROSOL A-102 was selected for further evaluation.

Run 117 used 60 g 10% PVA (VINOL 125), 4 g 25%  
10 glutaraldehyde, and 1.5 g 25 % p-toluene sulfonic acid emulsified in 100 g o-xylene containing 3 g PLURONIC L92 and ultrasonified using a Branson Ultrasonifier (setting 7; 50% duty cycle; 2 min.). The o-xylene/water azeotrope was stripped in a Buchler Rotovap rotary evaporator, and o-xylene was added to the emulsion until 45  
15 ml water and 185 ml o-xylene were removed. The final emulsion was then ultrasonified as before mentioned for this run, the catalyst was added, and the emulsion was tumbled end-over-end in capped bottles for 12 hours at room temperature.

The emulsion was then inverted by adding it to an excess  
20 of water containing 0.2% TRITON X-305 or AEROSOL A-102. The result was an aqueous dispersion of crosslinked poly(vinyl alcohol) particles.

Those skilled in the art will appreciate that the molecular weight of the poly(vinyl alcohol) will affect the properties  
25 of the crosslinked polymer particles. A high molecular weight polymer requires fewer crosslinks to crosslink than a low molecular weight polymer. The critical parameter is the molecular weight between crosslinks. Although, I do not want to be held to this theory, the molecular weight of a polymer that would give the desirable  
30 property of forming a worm-like thread upon extrusion from a hypodermic needle is probably one having a relatively low-to-moderate molecular weight.

Other experiments were conducted using Dow Methocel K4M methylcellulose. This is a hydroxypropylmethyl cellulose  
35 (commonly known as "methyl cellulose") and is representative of this

type of water-soluble derivative of natural cellulose. Methyl cellulose can be crosslinked by the reaction of its hydroxylgroups with such difunctional compounds as citric acid, glyoxal, dimethylolurea, water-soluble melamine-formaldehyde resins, quarternary ammonium salts, and water-soluble urea-formaldehyde resins.

Acetal-like derivatives of water-soluble partially-alkylated celluloses were produced by known techniques by treating an alkylated cellulose such as methyl cellulose with an aldehyde such as glyoxal or pyruvic aldehyde having the general formula  $R-CO-CO-H$ , in which R may be a hydrogen or alkyl group, and removing the water from the reaction products, e.g., by drying on a glass plate. The products obtained were insoluble in water, acetone, and low monohydric aliphatic alcohols.

The methyl cellulose was dissolved by wetting the powdered polymer at 75-85°C and then adding the remaining water while cooling and stirring. The clarity of the solution was improved by chilling to less than 10°C. 16 g 1% aqueous methyl cellulose was mixed in the conventional manner with 0.1 g hexamethoxymethyl melamine (CYMEL 300) and 0.0128 g p-toluene sulfonic acid catalyst and heated for 20 minutes at 50°C to form a crosslinked gel. When heated, instead, for 30 minutes at 60°C, a large mass of crosslinked polymer was formed. This crosslinking reaction was carried out in the emulsion.

50 g of a solution containing 1% methyl cellulose, 0.15 g CYMEL 300, and 0.070 g p-toluene sulfonic acid was emulsified in 50 g o-xylene containing 5.0 g SPAN 60. The emulsion was ultrasonified (setting 5; 10% duty cycle; 5 min.) to give an emulsion of 0.20-0.40 micrometers droplet size. The emulsion was then heated for 30 minutes at 70°C to crosslink the polymer droplets. Afterwards, the emulsion was then inverted in 800 ml water containing 10 g TRITON X-305 (HLB 17.3). Optical microscopy showed that the final emulsion, after being inverted in water was flocculated. Based on this observation, it was believed that a high HLB emulsifier might not be suitable for the inversion; accordingly, other such emulsifiers were tried.

TRITON X-102 (an octylphenoxy polyethoxy ethanol, HLB 14.6, available commercially from Union Carbide) in 9:1 water-emulsifier ratio did not invert the emulsion; TRITON N-101 (an nonylphenoxy polyethoxy ethanol, HLB 13.4, Union Carbide) gave similar results; TRITON X-405 (HLB 17.9) and TRITON X-305 (HLB 17.3) gave separation into two layers. Optical microscopy showed there was no flocculation with TRITON X-102 OR TRITON N-101, slight flocculation with TRITON X-405, and much more flocculation with the TRITON X-305.

In another experiment 50 g 1% aqueous methyl cellulose containing 0.25 g CYMEL 300 and 0.06 g p-toluene sulfonic acid was emulsified in 70 g o-xylene containing 7 g SPAN 60. The emulsion was subjected to hand-homogenization and ultrasonification, and then was stirred at 158 rpm in a round-bottom flask with a conventional laboratory stirrer for 2.5 hours at 60°C. 50 ml of this emulsion was inverted in 500 g water containing 5 g TRITON X-405 (HLB 17.9) to give an aqueous dispersion of the crosslinked methyl cellulose/CYMEL 300 particles.

A further experiment was conducted using 50 g 2.5% aqueous carboxymethyl cellulose solution containing 0.20 g CYMEL 300 and 0.06 g p-toluene sulfonic acid. This water-soluble polymer solution was emulsified in 50 g o-xylene containing 10% SPAN 60 and subjected to hand-homogenization and ultrasonification (setting 7; 50% duty cycle; 2 min), and heated for 1 hour at 62° C while being stirred at 240 rpm, to prepare an aqueous dispersion of crosslinked carboxymethyl cellulose particles.

Aqueous solutions of poly(vinyl alcohol) (10%) and methylcellulose (5%) each containing glutaraldehyde were emulsified in o-xylene containing 10% or 15% SPAN 60 in polymer solution/o-xylene ratios of 1:1 and 1:2 and allowed to react at room temperature in capped bottles. 10 g 5% methyl cellulose and 6.0 g (%) glutaraldehyde failed to react after 14 hours at room temperature or 30 minutes at 60°C. 10 g 5% methyl cellulose emulsified in 10 g o-xylene containing 10% SPAN 60, with and without 1.0 g glutaraldehyde, also failed to react at room

temperature. 10 g 5% methyl cellulose emulsified in 30 g o-xylene containing 15% SPAN 60, with and without 1.0 g 25% glutaraldehyde, gave slightly better results. Similarly, 20 g 15% PVA with 10 g 25% glutaraldehyde failed to react after 14 hours at room temperature.

Generally, the CYMEL 300 showed a significant solubility in the o-xylene as did the p-toluene sulfonic acid. At 40°C, the CYMEL 300 polymerized in o-xylene to form a precipitate. In contrast, glutaraldehyde did not dissolve in the o-xylene, even at 60°C. TETRONIC 1102 water-in-oil emulsifier was effective in emulsifying the methyl cellulose in o-xylene. TRITON X-405 (HLB 17.9) was effective in inverting these TETRONIC 1102-stabilized emulsions in water. Neither methyl cellulose nor PVA reacted with glutaraldehyde within 14 hours at room temperatures or 2 hours at 70°C. The combination of PVA or methyl cellulose and glutaraldehyde with a few drops of hydrochloric acid reacted after 1.5-2.0 hours at 100°C.

#### Crosslinking of Chondroitin Sulfate in Aqueous Solution

Tables XX and XXI show that the crosslinking of chondroitin sulfate with XAMA-7 crosslinking agent did not give the desired hydrophilic chondroitin sulfate gel (as it did with sodium alginate) even at the lower chondroitin sulfate/XAMA-7 weight ratios. Instead, a white, amorphous precipitate was formed. Table XX shows that decreasing the XAMA-7 concentration at constant chondroitin sulfate concentration (ph 7.5) so as to give chondroitin sulfate/XAMA-7 ratios of 2.0-4.0 also gave a white, amorphous precipitate. The amount of this precipitate decreased with increasing chondroitin sulfate/XAMA-7 ratio.

Table XX

Crosslinking of Chondroitin Sulfate  
with XAMA-7

Chondroitin Sulfate Run No.	(%)	CS (g)	Water* (g)	XAMA-7 (g)	CS/XAM-7 Ratio	Time Results**	(hr)
CSX-071	7	0.219	2.906	0.219	1.0	precipitation	>0.8
CSX-072	7	0.219	2.906	0.175	1.3	precipitation	>0.8
CSX-073	7	0.219	2.906	0.146	1.5	precipitation	>0.8
CSX-074	7	0.219	2.906	0.125	1.8	precipitation	>0.8
CSX-075	7	0.219	2.906	0.110	2.0	precipitation	>0.8
CSX-076	7	0.219	2.906	0.0735	3.0	precipitation	>24
CSX-077	7	0.219	2.906	0.0540	4.0	precipitation	>24
CSX-078	8	0.219	2.906	0.0438	5.0	precipitation	>24

\* pH 7.5

\*\* formation of white, amorphous precipitate; no visible gelation

20

This white, amorphous precipitate was attributed to the formation of a tight, inelastic gel by crosslinking of the low-molecular-weight chondroitin sulfate. This sample of chondroitin sulfate appeared to have a lower molecular weight than the sodium alginate or hyaluronic acid samples used earlier, and its 10% solution had a low viscosity.

When the sodium alginate was replaced with the chondroitin sulfate in the procedure used to prepare hydrophilic sodium alginate microspheres, the product resembled an inverse latex more than an inverse suspension. The molecular structure of chondroitin sulfate contains a -OSO<sub>3</sub>Na group in addition to the carbonyl and hydroxyl groups. This is the major difference between chondroitin sulfate, and hyaluronic acid and sodium alginate, which gave different results. When 3 g water was shaken with 3 g toluene in a small vial, and 0.15 g chondroitin sulfate was added, an oil-in-water emulsion was formed. At pH higher than 10, this effect was more pronounced. Thus, chondroitin sulfate is a special polysaccharide compared with hyaluronic acid and sodium alginate;

it acted as an emulsifier in alkaline solution because of the  $-\text{OSO}_3\text{Na}$  group.

Table XXI

Crosslinking of Chondroitin Sulfate  
with XAMA-7

Run No.	Chondroitin Sulfate (%)	Chondroitin Sulfate		XAMA-7 (g)	CS/ZAMA-7 Ratio	Results*
		CS (g)	Water* (g)			
CSX-082	8	0.250	2.875	0.1261	2.0	++++
CSX-083	8	0.250	2.875	0.0855	3.0	+++
CSX-084	8	0.250	2.875	0.0625	4.0	++
CSX-085	8	0.250	2.875	0.0500	5.0	+

\*pH 8.0

\*\* formation of white precipitate in >7 hours; no gelation

Crosslinking of Hyaluronic Acid in Aqueous Solution

Hyaluronic acid (0.14 g) was dissolved in 2.0 g water to give a 7% semi-solid transparent gel. This gel was diluted with 2.0 g water to give an immobile 3.5% gel. When 1.0 g of this 3.5% hyaluronic acid gel was diluted with 1.5 g water, it gave a viscous liquid that flowed slowly when inverted. XAMA-7 crosslinking agent (0.15 g) was added to give a hyaluronic acid/XAMA-7 ratio of 0.23; after 30 minutes, a white elastic gel was formed.

Table XXII shows that the pH range 7-9 was favorable for the crosslinking of hyaluronic acid, but that the pH range 12-13 was unfavorable. Table XXIII shows that hyaluronic acid/XAMA-7 ratios lower than 0.2-0.4 gave the best gels for the preparation of the crosslinked hyaluronic acid microspheres; ratios higher than 0.6-2.0 gave poorer gels that were not satisfactory for the microspheres, and ratios of 1.5-2.0 gave inelastic and immobile gels after 24 hours at room temperature.

Table XXIV, which used the lower-molecular-weight hyaluronic acid ( $0.75 \times 10^6$ ), showed different results. The higher-molecular-weight hyaluronic acid had to be used at a concentration

of 1.5%; higher concentrations gave immobile gels. The lower-molecular-weight hyaluronic acid was used at 2.5% concentration; this gave a mobile solution; higher concentrations such as 3.5% gave immobile gels. The optimum hyaluronic acid/XAMA-7 ratio for producing an elastogel was 0.5 for the lower-molecular-weight hyaluronic acid and 0.25 for the higher-molecular-weight. More XAMA-7 was required to crosslink the lower-molecular-weight hyaluronic acid to a suitable elastogel than for the higher-molecular-weight hyaluronic acid. This trend was expected: the lower the molecular weight, the greater the number of crosslinks required for insolubility. There may be some intramolecular crosslinking of the higher-molecular weight hyaluronic acid.

Table XXII

Crosslinking of Higher-Molecular-Weight\* Hyaluronic Acid  
with XAMA-7 at Different pH

Run No.	Hyaluronic Acid (HA)			pH	XAMA-7 (g)	HA/ XAMA-7 Ratio	Time to Cloud** (min)	Time to Gelation (min)
	(%)	HA (g)	Water (g/g)					
HAX-1513	1.5	0.0450	3.0025	13	0.0490	0.92	—	—
HAX-1512	1.5	0.0450	3.0011	12	0.0457	0.99	—	—
HAX-1511	1.5	0.0450	3.0078	11	0.0452	1.0	85	420
HAX-1510	1.5	0.0450	3.0000	10	0.0450	1.0	60	150
HAX-1509	1.5	0.0450	3.0000	9	0.0458	0.98	60	130
HAX-1508	1.5	0.0450	3.0010	8	0.0455	0.93	60	120
HAX-1507	1.5	0.0450	3.0021	7	0.0450	1.0	60	120

\*  $1.2 \times 10^6$

\*\* formation of a cloudy precipitate



Table XXIII

**Crosslinking of Higher-Molecular-Weight\* Hyaluronic Acid  
with XAMA-7 at Different HA/XAMA-7 Ratios at pH 8**

5

Run No.	Hyaluronic Acid (HA)			pH	XAMA-7 (g)	HA/ XAMA-7 Ratio	Time to Cloud** (min)	Time to Gelation (min)
	(%)	HA (g)	Water (g)					
HAX-15820	1.5	0.0458	3.0050	8	0.0229	2.0	>180	>1440***
10 HAX-15817	1.5	0.0454	3.0053	8	0.0270	1.75	100	>1440***
HAX-15815	1.5	0.0454	3.0021	8	0.0303	1.5	80	>1440***
HAX-15810	1.5	0.0456	3.0000	8	0.0454	1.0	60	120
HAX-15808	1.5	0.0456	3.0023	8	0.0563	0.8	60	120
HAX-15806	1.5	0.0456	3.0029	8	0.0750	0.6	60	120
15 HAX-15805	1.5	0.0459	3.0010	8	0.0900	0.5	60	120
HAX-15804	1.5	0.0460	2.9997	8	0.1121	0.4	45	elastogel
HAX-15802	1.5	0.0452	3.0003	8	0.2250	0.2	40	elastogel

\*  $1.2 \times 10^6$

\*\* time to form a cloudy, white precipitate

\*\*\* inelastic, movable gel

20

Table XXIV

**Crosslinking of Lower-Molecular-Weight\* Hyaluronic Acid  
with XAMA-7 at Different HA/XAMA-7 Ratios at pH 8**

Run No.	(%)	Hyaluronic Acid (HA)			XAMA-7 (g)	HA/ XAMA-7 Ratio	Gelation Cloud	Time** Gel
		(g)	Water (g)	pH				
25815	2.5	0.0769	3.000	8	0.0522	1.5	50	>180
25812	2.5	0.0769	3.000	8	0.0641	1.2	50	>180
25810	2.5	0.0769	3.000	8	0.0760	1.0	50	180
25808	2.5	0.0769	3.000	8	0.0983	0.8	50	130
25805	2.5	0.0769	3.000	8	0.1526	0.5	50	60***

\*  $0.75 \times 10^6$

\*\* in minutes

\*\*\* elastogel

**Crosslinking in Aqueous Emulsion**

Hyaluronic acid microspheres were prepared using the same procedure used to prepare the sodium alginate microspheres. First, ammonium hydroxide was added to raise the pH of the hyaluronic acid solution to 10-11; the solution was then emulsified in toluene to give the desired droplets. Then, the XAMA-7 crosslinking agent was added and the pH was decreased to 8-9 using acetic acid. The system was then allowed to crosslink at room temperature for 4 or 24 hours; then, the isopropanol dehydrating agent was added. The sample formed two layers: the top layer was separated and washed with methanol, to give a single phase without microspheres. The failure to produce microspheres was attributed to the 1.75 or 2.0 hyaluronic acid/XAMA-7 ratio and the high molecular weight of the hyaluronic acid ( $1.2 \times 10^6$ ). A 3.5% concentration of hyaluronic acid gave an immobile gel. A 1.5% solution of hyaluronic acid was viscous enough to make the inverse droplets in the presence of Span 60 emulsifier in toluene solution. This may be the major reason why low-concentration polymer solutions require a high concentration of crosslinking agent.

To prepare hydrophilic hyaluronic acid gel microspheres, 100 g aqueous solution containing 1.5 g hyaluronic acid was dispersed in 100 g toluene containing 1.0% w/w Span 60 using a mechanical stirrer at 1000 rpm for 15 minutes. Then, 6.0 g  
5 XAMA-7 crosslinking agent was added, the dispersion was stirred for another 5 hours, after which 100 ml isopropanol dehydrating agent was added to further harden the microspheres formed. The upper layer of toluene/isopropanol mixture was separated by decantation. The microspheres were washed twice with 200 ml  
10 isopropanol, separated by ultracentrifugation, and dried in an air oven at 75°C.

Table XXV shows that, for the higher molecular weight ( $1.2 \times 10^6$ ), the weight ratio of hyaluronic acid/XAMA-7 crosslinking agent and the pH of the system were important: hyaluronic  
15 acid/XAMA-7 ratios of less than 0.25 and control of the pH, first at 10-11 to prepare the emulsion, then at 8-9 for the crosslinking step, were required to give the desired microspheres. Hyaluronic acid/XAMA-7 ratios higher than 0.5, such as 1.25 or 1.75 (Runs HAB-01 and -02), did not give the desired microspheres. If the pH  
20 of the system was kept at 7-8, the microspheres became crosslinked, but easily stuck together.

Table XXVI shows that the lower-molecular-weight ( $0.75 \times 10^6$ ) hyaluronic acid gave spherical microspheres at a hyaluronic acid/XAMA-7 ratio of 0.5. Most of these spherical  
25 microspheres were smaller than 212 micrometers.

The molecular weight of the hyaluronic acid used in Table XXV ( $1.2 \times 10^6$ ) was greater than that used in Table XXVI ( $0.75 \times 10^6$ ). More XAMA-7 was required for crosslinking this  
30 higher-molecular-weight hyaluronic acid than for the lower-molecular-weight hyaluronic acid. This trend was the opposite of that expected and was attributed to the possible intramolecular crosslinking of the higher-molecular-weight hyaluronic acid. The desired elastic gel microspheres were obtained in both cases.

The drying of the microspheres was also important;  
35 after the microspheres were washed with the isopropanol

dehydrating agent, five drops of the suspension were placed on a glass slide and heated in an air oven at 75°C; when the water was evaporated, the microspheres were generally smaller in size, with smooth surfaces, as compared with those of the whole sample dried  
5 in the oven. The efficiency of the drying method must be investigated further. A fluidized bed dryer may be necessary to dry the microspheres on larger scale.

Table XXV

**Higher-Molecular-Weight\* Hyaluronic Acid Microspheres**  
**Crosslinked with XAMA-7**

5	Run No.	HAB-01	-02	-03	-04
	Water Phase (g)				
	Hyaluronic acid	1.5	1.5	1.5	1.5
10	Water	100	100	100	100
	pH**	7-8	7-8	7-8	10-12
	Oil Phase (g)				
	Toluene	100	100	100	100
15	Span	1.0	1.0	1.0	1.0
	Crosslinking Agent (ml)				
	XAMA-7	0.67	1.1	5.0	5.0
20		0.80 g	1.3 g	6.0 g	6.0 g
	HA/XAMA-7 Ratio	1.75	1.25	0.25	0.25
	pH**	7-9	7-9	7-9	7-9
25	Stirring Rate (rpm)	1000	800	1000	1000
	Reaction time (hr)	5	13	5	12
	Dehydrating agent				
30	Isopropanol (ml)	100	100	2x200	2x200
	Methanol (ml)	100	100	—	—
	Particle Shape	liquid	liquid	aggregated	spherical
35	Microspheres (%)				
	a. >150 $\mu$ m	—	—	—	11.5
	b. 150-212 $\mu$ m	—	—	—	12.6
	c. 212-150 $\mu$ m	—	—	69.7	68.8
	d. <150 $\mu$ m	—	—	30.3	7.0
40					

\*  $1.2 \times 10^6$ 

\*\* pH adjusted with concentrated ammonium hydroxide or acetic acid

Table XXVI

Lower-Molecular-Weight Hyaluronic Acid\* Microspheres  
Crosslinked with XAMA-7

5	<u>Run No.</u>	<u>LHAB-01</u>	<u>LHAB-02</u>
	Water phase (g)		
	LHA	2.5	2.5
10	Water	100	100
	pH**	10-11	10-11
	Oil Phase (g)		
	Span 60	1.0	1.0
15	Toluene	100	100
	Crosslinking Agent (ml)		
	XAMA-7	4.2	4.2
20		5.0 g	5.0 g
	LHA/XAMA-7 wt ratio	0.5	0.5
	pH**	8-9	8-9
25	Stirring rate (rpm)	1000	1000
	Reaction time (hr)	12	12
	Dehydrating agent (ml)		
30	Isopropanol	2x200	2x200
	Particle shape	spherical	spherical
	Microsphere size (%)		
35	a. >250 um	5.9	23.9
	b. 250-212 um	6.9	3.4
	c. 212-150 um	35.3	24.2
	d. <150 um	51.2	48.6

40 \* 0.75x10<sup>6</sup>

\*\* pH adjusted with concentrated ammonium hydroxide or acetic acid.

### Example 29

This example illustrates a 500 ml batch size production of microspheres.

5

#### 1. Prepare Solutions

- Solution A: Dissolve 15.00 g NaOH in 500 ml distilled and deionized water.
- 10 Solution I: Mix 0.5 g hyaluronic acid with 25 ml of Solution A in a 50 ml test tube.
- Solution B: Mix 9.0 g Sobitan monostearate (Span-60) in 150 ml toluene in a 500 ml glass bottle.
- 15 Solution II: Add 10 ml ethylene glycol diglycidyl ether (EGDGE), or other diepoxide type crosslinking agent, to Solution B.

#### 2. Cross-linking Reaction

- 20 Step 1: Pour Solution I in a 500 ml three-neck indented glass round bottom reactor, which was pre-equilibrated at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ .
- Step 2: Set stirring speed of a shaft agitator at 500 RPM  $\pm$  10 RPM; allow to stir continuously for 20 minutes  $\pm$  2 minutes.
- 25 Step 3: Add 0.5 ml of polyoxyethylene [20] sorbitan monooleate (Tween-80) into reactor.
- Step 4: Continue stirring for 10 minutes  $\pm$  1 minute, add 5.0 ml of cross-linking agent (EGDGE) into the reactor. (Note: this is additional to the cross-linking agent used in Solution II).
- 30 Step 5: Continue mixing for 10 minutes  $\pm$  2 minutes, then pour Solution II into the reactor.
- Step 6: Increase agitation speed immediately up to 1,000 RPM  $\pm$  50 RPM.
- 35 Step 7: Stop the agitation and cease the run at 9.0 hours  $\pm$  30 minutes.

#### 3. Washing, Purification & Sieving

- 40 Step 8: Add 300 ml methanol into the resulting gel suspension and continuously stir for 15 minutes  $\pm$  2 minutes at 1000 RPM  $\pm$  50 RPM.

Step 9: Stop the washing, carefully remove agitator apparatus from reactor solution, and then separate beads from gel solution by vacuuming filtration.

Step 10: Wash the beads with 400 ml acetone and continue vacuuming for 5 - 8 minutes.

Step 11: Reconstitute the beads with 2 liters of distilled deionized water and stir at 100 RPM, 20 - 24°C for overnight.

Step 12: Sieve the beads using the USA Standard Testing Siever to the desired size range from 20 to 90  $\mu$ m.

#### 4. Prepare For Coupling of Bioactive Molecules (optional)

Step a: Wash 10 ml of gravity-packed beads with 100 ml water 3 times.

Step b: Suspend the washed beads in 10 ml water and adjust pH to 4.0 - 4.5.

Step c: Dissolve 0.5 mg of Bioactive Molecule in 2.0 ml water and adjust pH to 4.0 - 4.5.

Step d: Add 2.5 mmol (about 500 mg) Coupling Agent to the beads' suspension and maintain the pH at 4.0 - 4.5.

Step f: Immediately add the solution of Bioactive Molecule into the bead suspension and maintain the pH at 4.0 - 4.5.

Step g: Place the bead suspension in a shaker and shake for 4 hours at room temperature.

Step h: Wash the gel sequentially with 2 x 100 ml water, 100 ml 0.1 M acetic acid, 100 ml water, 100 ml 0.1 M NaOH, 100 ml 0.5 M NaCl, and 100 ml PBS.

Step i: Store the bioactive molecule coupled beads in PBS containing anti-microbial agents at 4°C.

### **Example 30**

Polysaccharide microspheres were prepared according to the process of Example 29. Microspheres ranging in size from 10  $\mu$ m to 200  $\mu$ m were prepared using alginate of various formulations, chondroitin sulfate, hyaluronates with different molecule weights, inulin and cross-linked hydroxypropylmethyl cellulose (HPMC) - a "fiber-type" implant (i.e. not spherical) was also prepared according to the process of Example 29 using hydroxypropylmethyl cellulose. The size, rigidity and rheology characteristics of the microspheres were easily controlled by varying the process parameters.



### Protocol For Synthesis Of Cross-Linked Hydroxypropylmethyl Cellulose (HPMC)

- 5    Aqueous:    1 gram HPMC in 50 ml of 0.5M NaOH + 30 gram of  
EGDGE +3.2 grams of Tween 80  
Organic:    100 ml Toluene  
Reaction:    Mix organic into aqueous, after mixing well  
51.02 grams EGDGE are added.  
10            Stir overnight  
Final Reaction:    20 Ml of 0.5 M NaOH +5 gram EGDGE is  
added  
Heat the system to 70 deg. C for two hours  
Cool to room temperature  
15    Purification:    Wash with 1-propanol 3 times,  
wash with acetone 1 time  
wash with dionized water 3 times  
Depyrogenation:    Using 1.0 M NaOH overnight at room  
temperature  
20    Storage:    Depyrogenated PBS

### Protocol For Synthesis of Cross-Linked Sodium Hyaluronate Gel (General)

- 25 Aqueous solution: 1.0 g Sodium Hyaluronate in 50 mL of 0.75M NaOH +3 mL Tween 80
- Organic solution: 150 mL toluene +9 mL Sorbitan Triooleate (SPAN 85).
- 30
- Reaction: Add 25 mL Ethylene glycol diglycidyl ether (EGDGE) to aqueous solution when sodium hyaluronate was completely dissolved in aqueous solution in a reactor placed in waterbath at 70°C. Organic solution was added after EGDGE dissolved in aqueous solution. The reactor was cooled down to room temperature
- 35

30 min after addition of organic solution. Add 75 mL EGDGE and continue stirring overnight.

Purification: Was in 1-propanol (3 times gel volume) 3 times, acetone (3 times gel volume) 2 times, distilled H<sub>2</sub>O (5 times gel volume) 2 times. The beads were boiled in distilled H<sub>2</sub>O for 30 minutes.

Protocol for Synthesis of Cross-Linked Chondroitin Sulfate A Gel  
(General)

Aqueous solution: 1.0 Chondroitin Sulfate A in 50 mL of 0.75 M NaOH +3 mL Tween 80

Organic solution: 150 mL toluene +9 mL Sorbitan Triooleate (SPAN 85)

Reaction: Add 25 mL Ethylene glycol diglycidyl ether (EGDGE) to aqueous solution when chondroitin sulfate A was completely dissolved in aqueous solution in a reactor placed in waterbath at 70°C. Organic solution was added after EGDGE dissolved in aqueous solution. The reactor was cooled down to room temperature 30 min after addition of organic solution. Add 75 mL EGDGE and continue stirring overnight.

Purification: Wash in 1-propanol (3 times gel volume) 3 times, acetone (3 times gel volume) 2 times, distilled H<sub>2</sub>O (5 times gel volume) 2 times. The beads were boiled in distilled H<sub>2</sub>O for 30 minutes.

Protocol For Making Inulin Beads (General)

Aqueous solution: 1.0g Inulin in 50 mL of 1M NaOH +3 mL Tween 80

Organic solution: 200 mL toluene +12 mL SPAN 85 (Sorbitan Trioleate) +20 mL EGDGE (Ethylene glycol diglycidyl ether)

Reaction: Add 35mL of EGDGE to solution I, then 178 mL of  
5 solution II was immediately added to solution I after EGDGE dissolved in solution I. Stir for ~ 40 hrs.

Purification: Wash in 1-propanol (2 times gel volume 4 times,  
acetone (2 times gel volume) 2 times, distilled H<sub>2</sub>O (5 times gel  
10 volume) 5 times. The beads were boiled in distilled H<sub>2</sub>O for 30 minutes.

Depryogenation: Using 0.5M NaOH, overnight at room temperature.

15

Protocol for Synthesis of Cross-Linked Alginate Gel (General)

Aqueous solution: 1.0g Alginate in 50 mL of 0.5M NaOH +3 mL  
Tween 80

20

Organic solution: 150 mL toluene +6 mL Sorbitan Trioleate (SPAN 85)

Reaction: Add 10 mL of Ethylene glycol diglycidyl ether (EGDGE)  
25 to aqueous solution when alginate was completely dissolved in aqueous solution in a reactor placed in waterbath at 70°C. Organic solution was added after EGDGE dissolved in aqueous solution. The reactor was cooled down to room temperature 30 min after addition of organic solution. Add 30 mL EGDGE and continue stirring  
30 overnight

Purification: Wash in 1-propanol (3 times gel volume) 3 times,  
acetone (3 times gel volume) 2 times, distilled H<sub>2</sub>O (5 times gel  
volume) 2 times. The beads were boiled in distilled H<sub>2</sub>O for 30  
35 minutes.

**Example 31**

The microspheres produced in Example 30 passed cytotoxicity, sterility and pyrogenicity tests and were found to be substantially non-toxic.

**Example 32**

Immunogenicity tests were performed on two different batches of alginate microspheres and on the hyaluronate microspheres produced in Example 31 by implanting in animals. These microspheres elicited only a minimal immunological response in implanted animals.

**Example 33**

An acute inflammation test was performed on microspheres produced according to Example 30. All materials were injected easily into the peritoneal cavity of mice. No mortality, morbidity, signs of toxicity, or any signs of distress were observed in the test animals. The test revealed that the type, but not shape, of implant material affects the short term inflammatory cell responses of the host to the implants. Hydroxypropylmethyl cellulose gels recruit more neutrophils than other materials. Hyaluronate microspheres attract more transient monocytes/macrophages than other materials tested.

**Example 34**

Chronic implantation tests using the microspheres of Example 32 showed no adverse systemic or local tissue reaction to the implants. The microspheres retained their shape and size in the implant site.

### Example 35

It was found that the microspheres of Example 32 were easily extruded from 22 to 28 gauge hypodermic needles and delivered into tissues. No surgical incision or complicated procedure was necessary for implantation. The implant and surrounding tissue showed no sign of redness, irritation or swelling.

### Example 36

10

In this example, the batch size was 22 liters, the hydrophilic polymer was hyaluronic acid, the organic phase was toluene, the emulsifying agents were a combination of SPAN 60 and TWEEN 80, and the crosslinking agent was ethylene glycol deglycidyl ether (a diepoxide type crosslinker that crosslinks through OH groups).

The agitation speed was only 400 rpm (a speed fast enough to mix the reagents, permitting the formation of a stable emulsion, but not so fast as to create excessive mechanical stress and cause shearing and crosslinking breakage).

The reaction was run at 25°C and the pH was adjusted to 7.4 after crosslinking. The reaction time was 8.5 hours followed by washing with methanol, acetone and then resuspension in phosphate buffered saline.

The reaction yielded particles having a size range of 10  $\mu\text{m}$  to 50  $\mu\text{m}$ , with approximately 75% of the particles being approximately 30  $\mu\text{m}$ .

The microparticles exhibited excellent stability. Under constant high shear force generated by agitation at 34.0 liter/sec mixing for 2 hours, the apparent viscosity changed only slightly (about 2.9%). The viscosity would drop significantly (e.g. more than 10%) if the particles break down into smaller particles and particle fragments.

The particles formed in this example were surprisingly easy to extrude through a 22 gauge needle, requiring less than 5 pounds per square inch.

5

### Example 37

We suspended Sephadex beads of different sizes and rigidities in either non-crosslinked hyaluronate in saline (1 mg/ml) or plain saline solutions and compared the extrusion force between  
10 the two. We found no difference between Sephadex suspensions with or without hyaluronate, demonstrating that the hyaluronate does not function as a delivery aid when mixed with injectable microspheres.

Even though non-crosslinked hyaluronate did function as an injection aid, it was found to be an useful ingredient for injectable  
15 implants because it reduced host inflammatory responses to implant materials. Thus, materials such as Sephadexs or any other crosslinked polysaccharide microspheres which may not be inherently biocompatible to the host, may be employed as implant materials when used in conjunction with hyaluronate.

20 It is postulated that the hyaluronate may "mask" the implant microsphere material, thereby preventing the host inflammatory cells (i.e., neutrophils, macrophages and monocytes) from recognizing and responding immunologically to them. Subsequent to implantation, when the hyaluronate likely is degraded,  
25 the tissue regeneration phase has begun and inflammatory cells may no longer play a role at the implant site.

I. Objective - To hydrate Sephadex beads in a solution of  
30 hyaluronate in PBS equal to 1 mg. hyaluronate per ml. of PBS.

II. Purpose - To add lubrication to the beads to ease flow through the needle.

III. Equipment

35 Instron 4200 series Interface with Load  
Cell#2418-80  
Upper Compression plate for Instron  
30cc Syringes (two for each test)

3cc Syringes - Becton Dickinson Cat #9585 (one  
for each 3cc of material for testing)  
22G/1 inch needles - Becton Dickinson Cat #5155  
Drying stand for transurethral needles  
5 Analytical Balance (RR-0026/Cal. due 7-29-96)  
Test tube rack  
Rubber stoppers for syringes  
Male/male lure-lok fittings

10 IV. Testing Materials

Sephadex G-200-50 - Sigma (lot# 64H1244)  
Sephadex G-75-50 - Sigma (lot# 75H907)  
Sephadex G-50-50 - Sigma (lot# 124H0078)  
Sephadex G-25--50 - Sigma (lot# 12H1057)  
15 Dulbecco's Phosphate Buffered Saline without calcium  
chloride and magnesium chloride  
Sigma - Cat #D-5527, Lot #44H4646, Exp. 05/96  
Sodium Hyaluronate  
Life Core - Cat #1011-1100, Lot# 1-91323-1

20

V. Procedure

- A. Make up hyaluronate solution in PBS  
1) 0.210 gm. hyaluronate added to 210 ml. of PBS  
2) Place on stir plate and stir with magnetic stirrer until  
25 completely in solution.
- B. Prepare 30cc syringes.  
1) Remove plunger  
2) Stopper bottom with rubber stopper  
3) Label with type of Sephadex to be added
- 30 C. Add 10cc of appropriate solution to each syringe.  
D. Weigh out 0.5 gm. of Sephadex for each syringe and add  
to syringe.
- E. Bring to 30cc volume with appropriate solution.  
F. Gently seat plunger back in place.
- 35 G. Invert syringe, remove rubber stopper and push out  
trapped air.
- H. Replace stopper and let stand on plunger in test tube  
rack overnight.
- I. Record total amount of swelled beads.
- 40 J. Remove stopper and push out liquid portion that does  
not contain Sephadex.
- K. Hook Male/Male fitting on end of syringe.

- L. Hook second 30cc syringe to sample.
- M. Mix sample by pushing back and forth between the two syringes five times.
- N. Remove second 30cc syringe.
- 5 O. Use fitting to transfer sample to 3cc syringes, 3cc per syringe.
- P. Place 22 G needle on each syringe of sample.
- Q. Test on extrudability on the Instron machine.

## 10 VI. Results -

### SEPHADEX/HYALURONATE

SAMPLE SEPHADEX	PLUS OR MINUS HYALURONATE	PACKED BEAD VOL. IN CC'S	NUMBER OF SYRINGES	MAXIMUM LOAD IN LBS. MEAN (STD. DEV.)	AVG. LOAD BETWEEN LIMITS MEAN (STD. DEV.)
G-200-50	PLUS	15	5	1.221 (.143)	0.6657 (.0512)
G-200-50	MINUS	16	5	1.269 (.109)	0.5877 (.1444)
G-50-50	PLUS	6	2	1.299 (.053)	0.8455 (.0980)
G-50-50	MINUS	7	1	1.224 (***)	1.019 (***)
G-25-50	PLUS	3	1	21.2 (***)	3.363 (***)
G-25-50	MINUS	3	1	1.272 (***)	0.78 (***)
G-75-50	PLUS	9	3	1.174 (.090)	0.6244 (.0843)
G-75-50	MINUS	9	2	1.224 (.016)	0.774 (.0129)

15

## VII. Conclusion

The hyaluronate at this level appeared to have little or no effect on the Sephadex. A higher concentration of hyaluronate  
 20 may give a more positive result.

### Example 38

This 30-day implantation study showed that the smaller  
 25 size microspheres trigger less inflammatory responses and therefore are more desirable than larger size microspheres when used as an injectable implant.



### Overall Purpose:

The objective of this study was to investigate whether the particle size of implanted polysaccharide microspheres influences long term  
5 inflammatory and/or fibrotic responses in a rat model.

### Animals:

Male Sprague Dowley rats in good health, weighing approximately  
10 100 grams, were used in the study.

### Test Materials and Animal ID Number

1. Test material #A (animal A1):  
15 Hyaluronic Gel (<20 - >150  $\mu$ m bead size)
2. Test material #B (animals B1 and B2):  
Hyaluronic Gel (20 - 45  $\mu$ m bead size)
3. Test material #C (animals C1 and C2):  
Hyaluronic Gel (45 - 33  $\mu$ m bead size)
- 20 4. Test material #D (animals D1 and D2):  
Hyaluronic Gel (63 - 90  $\mu$ m bead size)
5. Test Material # E (animals E1 and E2):  
Hyaluronic Gel ( 90 -  $\geq$ 150  $\mu$ m bead size)

### 25 Test Protocol:

1. Male Sprague Dawley rats (1 animal for test material #A, 2 animals per group for the other test materials, total 9 rats) were purchased Taconic Farms (Germantown, NY) and kept in the Albany  
30 Medical College Animal Resource Facility for 7 days prior to initiation of this study.

2. Rats were anesthetized with ketamine-xylazine (ketamine 80 mg/kg body weight and xylazine 12 mg/kg body weight) according to  
35 standard procedures. The fur on the back was then clipped.

3. 0.5 ml of test material was injected (by subscapular inoculation) into both the left and right sides of each animal.

4. After 30 days, rats were sacrificed by inhalation of CO<sub>2</sub>. The implanted materials and the tissues surrounding them were recovered by careful dissection and fixed with Formalin.

5

5. The fixed material was embedded *en bloc* in paraffin, sectioned and stained with haematoxylin and eosin.

#### Results:

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##### A. Material Injected:

Approximately 0.5 ml of the test materials were subscapularly administered to both left side and right side of animal. The weights of injected materials were measured and are listed in Table 1.

15

##### Weight of test Materials Implanted in Rats

	<u>ID#</u>	<u>Left-WT(grams)</u>	<u>Right-Wt(grams)</u>
20	A-1	0.62	0.59
	B-1	0.61	0.53
	B-2	0.65	0.59
	C-1	0.58	0.54
	C-2	0.48	0.53
25	D-1	0.46	0.55
	D-2	0.53	0.59
	E-1	0.61	0.53
	E-2	0.49	0.52

##### 30 B. Post Injection Animal Care

After implantation, the animals were observed for visible tissue responses around the implantation site. These were recorded at 1, 24, 72, and 144 hours post injection. The injection sites were scored on a scale of 0 to 3 as follows:

35

0 = No reaction

1 = Slight redness and swelling only over implant

2 = Mild redness and swelling over implant and surrounding tissues

3 = Severe redness and swelling over implant and surrounding tissues

5

### Visible Tissue Responses to Implanted Test Materials

Test Animal		The score of tissue responses materials			
#		1 hour	24 hours	72 hours	144 hours
10	A 1	0	0	0	0
	B 1	0	0	0	0
	B 2	0	0	0	0
	C 1	0	0	0	0
	C 2	0	0	0	0
15	D 1	0	0	0	0
	D 2	0	0	0	0
	E 1	0	0	0	0
	E 2	0	0	0	0

20 No externally visible inflammatory responses or other adverse tissue responses were observed.

### C. Animal Weights

25 The weights of animals before and 30 days after implantation were measured. The test materials had no obvious effect on the growth of the test animals.

### Weights and Weight Change in Implanted Animals

5	Rat ID#	Body weight (grams)		Gain	S.
		Initial	Final		
	A-1	95.1	327.4	232.3	
	B-1	84.5	311.7	227.2	
	B-2	102.6	349.6	247.2	
10	C-1	108.6	333.5	224.9	
	C-2	100.6	334.1	233.5	
	D-1	99.5	294.8	195.3	
	D-2	94.4	324.7	230.3	
	E-1	100.4	338.6	238.2	
15	E-2	98.4	308.6	210.2	

#### D. Histopathology:

After 30 days implantation, the materials implanted  
 20 were recovered, fixed and embedded as described above. The  
 embedded samples were sectioned and stained with haematoxylin and  
 eosin. The tissue responses were graded according to the extent of  
 foreign body response (primarily, the numbers of foreign body giant  
 cells present), fibrosis and extent of cellular infiltration (on a scale of  
 25 0 to 3):

- 0 = No visible response
- 1 = Weak response
- 2 = Mild response
- 30 3 = Strong response

Note: The grade 0 - 3 was designed for comparison purpose in  
 order to differentiate the 5 studied materials. The interpretations for  
 each test material (below) are also with the same purpose. There was  
 35 no appearance of any adverse reaction in animals during a 30-day  
 implantation with any of 5 polysaccharide materials. All 5 materials  
 are considered safe for tissue implantation.

### Type and extent of Tissue Reactions to Implants

	Test materials	Samples #	Foreign Body Responses	Fibrosis	Cell Filtration
5	A	1	3+	1-2+	3+
	A	2	3+	1+	3+
10	B	1	1-2+	2-3+	1+
	B	2	1+	3+	0-1+
	C	1	1-2+	1-2+	1-2+
15	C	2	1-2+	2+	1-2+
	D	1	2+	2+	2+
	D	2	2-3+	2+	2-3+
20	E	1	3+	2+	3+
	E	2	3+	1-2+	3+

#### Test Material A

25           The foreign body reaction and chronic inflammatory response were quite evident around the implants. Significant fibrotic tissue (fibroblasts and collagen fibrils) was present in the capsules around the implant. Large numbers of foreign body giant cells and macrophages had penetrated the body of implants and enveloped  
30 individual particles of the test material.

#### Test Material B

35           These implants triggered only weak foreign body reactions. Strong fibrotic responses, notably an accumulation of large numbers of fibroblasts and collagen fibrils, were present. However, the cell material interaction was limited to the interface between and tissue. Cell penetration into the interstices of the implant body was minimal.

### Test Material C

Mild foreign body responses and fibrotic responses were observed around the implants. Foreign body giant cells were abundant in the material/tissue interface. Mild cellular infiltration also occurred immediately surrounding the implants. However, most of the inner portions of the implant were free of invading cells.

### Test Material D

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The implants prompted mild peripheral foreign body and fibrotic responses. However, a significant number of foreign body giant cells and macrophages penetrated the material implants. Notably, small blood vessels (angiogenesis) had developed within the implants.

15

### Test Material E

Strong foreign body reactions and mild fibrotic responses were found in the tissue immediately adjacent to these implants. Numerous foreign body giant cells occurred in most areas within and adjacent the implant.

20

### Conclusion:

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After implantation for 30 days, the different test materials triggered distinct tissue responses such as foreign body responses, fibrosis and cell infiltration. Test materials B and C triggered weak inflammatory response and mild fibrotic responses. In contrast, test materials A and E prompted marked inflammatory responses. The smaller size beads trigger only weak inflammatory responses and therefore are more desirable than larger size beads, which illicit a strong immunological response, when used for implantation.

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### Example 39

#### Study of Acute Inflammatory Responses To Implanted Polysaccharide Biomaterials in Mice

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##### Overall Purpose:

The objective of this study is to investigate whether different crosslinked polysaccharide materials made according to the process of the present invention cause acute inflammatory responses in mice.

10

##### Test Materials

- 15 1. Test material #1: Hydroxypropymethyl Cellulose Fiber (length 150 - 500  $\mu\text{m}$ , width 10 -20  $\mu\text{m}$ )
2. Test material #2: Inulin Beads (38 - 90  $\mu\text{m}$ )
3. Test material #3 : Alginate Beads (38 - 90  $\mu\text{m}$ )
4. Test material #4: Chondroitin Sulfate Beads (38 - 90  $\mu\text{m}$ )
- 20 5. Test material #5: Hyaluronic Acid Beads ( 38 - 90  $\mu\text{m}$ )
6. Saline
7. Zymosan A (1 mg/ml, from *Saccharomyces cerevisiae*)
- 25

##### Test Protocol

- 30 1. Male Balb/c mice were purchased from Taconic farm (Germantown, NY) and acclimated for 7 days prior to the initiation of this study.
2. Followed the implantation procedure, 1 ml of test materials was injected into mice peritoneums.

35

3. After implantation for 24 hours, mice were sacrificed and the peritoneal cells were then recovered with 5 ml of phosphate buffer saline.

5 4. The numbers of inflammatory cells (especial neutrophils and monocytes/macrophages) and total cells were determined by the cell specific enzyme activities.

### Results:

10

#### A. Animal Record

Test Materials	Animal #	Body Weight Before Implantation	Body Weight After Implantation	Total Weight Injected
15				
1	1	21.84 grams	19.83 grams	1.012 grams
1	2	20.24 grams	18.46 grams	1.011 grams
1	3	20.63 grams	18.58 grams	1.029 grams
20				
2	1	21.04 grams	19.24 grams	1.095 grams
2	2	20.00 grams	18.27 grams	1.125 grams
2	3	19.73 grams	18.30 grams	1.242 grams
2	4	21.94 grams	20.21 grams	1.191 grams
25				
3	1	18.39 grams	16.26 grams	1.041 grams
3	2	20.64 grams	18.76 grams	1.093 grams
3	3	20.18 grams	18.32 grams	1.066 grams
30				
4	1	21.14 grams	18.36 grams	1.029 grams
4	2	20.46 grams	18.09 grams	1.200 grams
4	3	22.23 grams	19.44 grams	1.033 grams
35				
5	1	21.04 grams	19.12 grams	1.073 grams
5	2	20.52 grams	18.02 grams	1.093 grams
5	3	17.73 grams	16.03 grams	1.042 grams
40				
6	1	20.48 grams	20.60 grams	1.081 grams
6	2	21.10 grams	20.93 grams	1.021 grams
6	3	21.98 grams	21.42 grams	1.001 grams
45				
7	1	20.75 grams	19.87 grams	1.052 grams
7	2	20.22 grams	19.77 grams	1.095 grams
7	3	20.95 grams	20.55 grams	1.042 grams

---

Brief observation:



Acute inflammatory responses to implanted biomaterials may cause the weight loss. However, it is difficult to simply determine the degree of inflammatory responses by the data of weight loss. Consequently, immunological cell numbers were estimated.

#### B. The recruitment of neutrophil (PMN)

Test Material	Myeloperoxidase activities (mUnit)	Estimated Cell Number ( $\times 10,000$ )
#1	$47.6 \pm 14.0$	$207.4 \pm 61.0$
#2	$12.8 \pm 6.5$	$55.8 \pm 28.3$
#3	$23.6 \pm 8.2$	$102.8 \pm 35.7$
#4	$18.0 \pm 12.8$	$78.4 \pm 55.8$
#5	$15.2 \pm 12.4$	$66.2 \pm 54.0$
#6	$0.0 \pm 0.0$	$0.0 \pm 0.0$
#7	$33.1 \pm 14.2$	$144.2 \pm 61.9$

#### Brief Interpretation:

Implanted foreign materials often found to trigger the acute inflammatory responses, which mediate the accumulation of inflammatory cells (neutrophils and macrophages) in implant sites. Naive or saline injected peritoneal cavity, the implantation site for this study, only a few neutrophils accumulated. After introduced with poor compatible materials (such as zymosan A-material #7), mice developed acute inflammatory responses with the accumulation of a large number of neutrophils. Therefore, the number of recruited neutrophils may be obtained and used as an indicator of the tissue compatibility to implanted materials. Because neutrophils can cause damage to the surrounding tissues and the implanted materials a good material implant shall prompt minimal influx of neutrophils. Material #1 caused prompt significant neutrophil accumulation. Moderate neutrophils accumulation was found for material #3 and only mild neutrophils accumulation was found using material #2, #4 and #5. Material #2, #3, #4 and #5 are considered to be acceptable as implant materials.

#### C. The recruitment of monocytes/macrophages

100

	<u>Test Material</u>	<u>Enzyme Activities</u> (mUnit)	<u>Estimated Cell Number</u> ( $\times 100,000$ )
5	1.	354 $\pm$ 48	322 $\pm$ 44
	2.	284 $\pm$ 75	258 $\pm$ 68
	3.	236 $\pm$ 9	215 $\pm$ 8
	4.	321 $\pm$ 25	292 $\pm$ 23
	5.	887 $\pm$ 466	807 $\pm$ 424
10	6.	31 $\pm$ 6	28 $\pm$ 5
	7.	48 $\pm$ 13	44 $\pm$ 12

### Brief Interpretation

- 15 About 2,000,000 resident macrophages usually reside in the naive mouse peritoneum. During inflammatory responses, peripheral monocytes may be recruited to the peritoneum to join the resident macrophages. Macrophages are important in mediating wound healing responses, for example phagocytosing tissue debris
- 20 and mediating the proliferation of fibroblasts,. Therefore, the number of macrophages recruited can be used as an indication of wound healing responsiveness, i.e. an indicator of tissue reconstruction occurring in the implant sites. Naive or saline (material #6) injected peritoneal cavity had 'normal' macrophages
- 25 (mostly resident macrophages) accumulation. Strong inflammatory agents (such as zymosan A-material #7) trigger severe inflammatory responses and the accumulation of neutrophils, which delay the appearance of macrophages. Material #5 triggered the most macrophage accumulation. Mild macrophages accumulation is found
- 30 on material #1, #2, #3 and #4. This study suggest that material #1, #2, #3, #4, and #5 do not impair normal wound healing responses.

**D. Total peritoneal cells**

<u>Test Material</u>	<u>Enzyme Activities</u> (mUnit)
1.	1526 $\pm$ 135
2.	397 $\pm$ 98
3.	877 $\pm$ 131
4.	924 $\pm$ 145
5.	1097 $\pm$ 593
6.	167 $\pm$ 155
7.	525 $\pm$ 155

**15 Brief Interpretation:**

In the process of implant mediated tissue responses, the implant sites are occupied by inflammatory cells (such as macrophages/monocytes and neutrophils) and proliferating fibroblast. The total number of cells in the implant sites, (peritoneal cavity), may represent the degree of tissue responses to implanted test materials. The total number of cells can be assessed by the activities of lactate dehydrogenase, which is a common cytoplasmic enzyme. Material #1 triggered substantial cell accumulation. Other test materials #2, #3, #4 and #5 induce milder tissue responses.

**Chronic Inflammatory and Fibrotic Responses To Implanted Polysaccharide Biomaterials in Rats**

30

**Overall Purpose**

The objective of this study was to investigate whether test polysaccharide materials differ in their tendency to trigger chronic tissue responses (including fibrosis and inflammation) in rats.

35

**Animals**

Male Sprague Dowley rats in good health, weighing approximately 150 grams, were used.

#### Test Materials and Animal ID Number

- 5
1. Test material #1 (1-1, 1-2, 1-3) Hydroxypropylmethyl Cellulose Fiber (length 150-500  $\mu\text{m}$ , width 10-20  $\mu\text{m}$ )
  2. Test material #2 (2-1, 2-2, 2-3) Inulin Beads (38-90  $\mu\text{m}$ )
  3. Test material #3 (3-1, 3-2, 3-3) Alginate (high  
10 mannuronic acid content) Beads 38-90  $\mu\text{m}$ )
  4. Test material #4 (4-1, 4-2, 4-3) Chondroitin Sulfate Beads (38- 90  $\mu\text{m}$ )
  5. Test material #5 (5-1, 5-2, 5-3) Hyaluronic Acid Beads (38-90  $\mu\text{m}$ )
  - 15 6. Test material #6 (6-1, 6-2, 6-3) Alginate (high guluronic acid content) Beads (38-90  $\mu\text{m}$ )
  7. Saline #8 (control) (7-1, 7-2, 7-3)

#### Test Protocol

- 20
1. Male Sprague Dawley rats (3 animals per test material, total 24 rats) were purchased from Taconic Farms (Germantown, NY) and acclimated for 7 days prior to initiation of this study.
  2. Rats were anesthetized with ketamine-xylazine (ketamine 80  
25 mg/kg body weight and xylazine 12 mg/kg body weight) according to standard procedures. The fur on the back was then removed with electric clippers.
  3. 1 ml of test material was implanted by subscapular inoculation in each animal.
  - 30 4. After 4 weeks the rats were sacrificed and the implanted materials with surrounding fibrous/collagenous tissues were carefully removed and fixed with buffered formalin.
  5. The explanted and fixed tissues were then embedded in paraffin, sectioned and stained with haematoxylin and eosin.

**Results:****A. Material Injected:****5 Weight of Test Materials Implanted in Rats**

	ID#	WT(grams)	ID#	WT(grams)	ID#	WT(grams)
	1-1	1.07	1-2	1.09	1-3	1.04
10	2-1	1.04	2-2	1.01	2-3	0.95
	3-1	1.02	3-2	1.03	3-3	1.04
	4-1	1.04	4-2	0.99	4-3	0.97
	5-1	1.04	5-2	1.00	5-3	1.04
	6-1	1.03	6-2	1.13	6-3	1.03
15	7-1	1.01	7-2	0.95	7-3	1.07

**B. Post-Injection Animal Care**

20 After the injection, the implanted animals were observed for visible tissue responses around the implantation site. These were recorded at 1, 24, 72, and 144 hours post injection. The injection sites were scored on a scale of 0 to 3 as follows:

- 25
- 0 = No reaction
  - 1 = Slight redness and swelling only over implant
  - 2 = Mild redness and swelling over implant and surrounding tissues
  - 3 = Severe redness and swelling over implant and surrounding tissues

### Visible Tissue Responses To Implanted Test Materials

5	Test materials	Animal #	The score of tissue responses			
			1	24 hours	72 hours	144 hours
	1	1	0	0	0	0
	1	2	0	0	0	0
	1	3	0	0	0	0
10	2	1	0	0	0	0
	2	2	0	0	0	0
	2	3	0	0	0	0
	3	1	0	0	0	0
	3	2	0	0	0	0
15	3	3	0	0	0	0
	4	1	0	0	0	0
	4	2	0	0	0	0
	4	3	0	0	0	0
	5	1	0	0	0	0
20	5	2	0	0	0	0
	5	3	0	0	0	0
	6	1	0	0	0	0
	6	2	0	0	0	0
	6	3	0	0	0	0
25	7	1	0	0	0	0
	7	2	0	0	0	0
	7	3	0	0	0	0

30 No evident tissue inflammatory responses were observed for all polysaccharide implants.

### C. Animal Weights

35 The weights of animals before and after implantation were measured. All animals gained weight after implantation and test materials had no obvious influence on the rate of growth as compared with the control group (#7).

### Weights and Weight Change in Implanted Animals

	Rat ID#	Body weight (grams)			Rat ID#	Body weight (grams)		
		Initial	Final	Gain		Initial	Final	Gain
5	1-1	138.6	365.3	226.7	2-1	172.4	368.3	195.9
	1-2	142.1	326.1	184.0	2-2	132.6	324.2	191.6
	1-3	160.9	365.1	204.2	2-3	138.9	341.8	202.9
10	3-1	150.4	337.2	186.8	4-1	152.3	333.5	181.2
	3-2	146.7	306.5	159.8	4-2	172.3	379.3	207.0
	3-3	160.8	384.1	223.3	4-3	136.1	309.9	173.8
15	5-1	170.6	379.2	208.6	6-1	157.8	354.3	196.5
	5-2	145.6	330.7	185.1	6-2	166.4	375.3	208.9
	5-3	160.6	341.1	180.5	6-3	138.8	345.2	206.4
20	7-1	163.8	364.6	200.8				
	7-2	147.8	357.4	209.6				
	7-3	146.3	344.4	198.1				

#### D. Histopathology

25

After 30 days of implantation, the material implants were recovered, fixed and embedded as described above. The samples were sectioned and stained with haematoxylin and eosin. The tissue responses to test materials were categorized into two patterns and subjectively graded according to increasing severity (0-3) by two investigators and by a board certified pathologist.

30

Pattern A: Higher fibrotic responses, typified by the presence of fibroblasts and fibrous material surrounding the implant mass. Strong inflammatory cell infiltration and giant cell reaction surrounding individual particles of test materials.

35

Pattern B: Weak cell filtration (centers of the implants have few or no cells) and relatively mild giant cell reactions and fibrosis around test materials.

40

## Type and Severity of Tissue Reactions to Implants

	Test Material	Animal #	Pattern	Foreign	Fibrosis body giant cells	Chronic Inflammation
5	1	1	B	3+	0	2+
	1	2	B	3+	0	1-2+
10	1	3	B	3+	0-1+	1+
	2	1	A	3+	2+	1+
	2	2	A	3+	2+	1+
	2	3	A	3+	0-1+	1-2+
15	3	1	-		(no material observed)	
	3	2	B	2+	1-2+	2+
	3	3	B	2+	2+	1-2+
20	4	1	A	3+	1-2+	1+
	4	2	A	3+	1+	1+
	4	3	A	3+	1+	1+
	5	1	A	3+	1+	0-1+
25	5	2	A	3+	1+	1+
	5	3	A	3+	1+	2+
	6	1	A	3+	1+	1+
	6	2	A	3+	2+	1+
30	6	3	A	3+	2+	0-1+
	7	1	B	0	0	0
	7	2	B	0	0	0
35	7	3	B	0	0	0

There was no appearance of any adverse reaction in animals during a 30-day implantation with any of 6 polysaccharide materials. All 6 materials are considered safe for tissue implantation. All implants (except animal 1 with material #3) were remained in the place when retrieving.



### Test Material #1

Foreign body reaction and chronic inflammatory response  
5 were evident around the material implants. No obvious fibrotic tissue (fibroblasts and collagen fibril) was found in the capsules around the implant. Most of the inflammatory cells accumulated around the implants, and few cells were found inside the implants.

### 10 Test Material #2

These implants triggered strong foreign body reactions and fibrotic responses. The implants were also surrounded by fibrous/collagenous capsules (100 to 300  $\mu\text{m}$  thick) comprised of  
15 collagen fibrils and numbers of foreign body giant cells and fibroblasts. The interstices of the implants also were filled with large numbers of cells. The invading cells (including numerous giant cells and some fibroblasts) were found encircling individual particles of test material.

20

### Test Material #3

This material initiated mild foreign body and fibrotic responses. A large number of fibroblasts, collagen fibrils and some giant cells  
25 formed fibrotic capsules around the implants (50-300  $\mu\text{m}$  thickness). Cell penetration to the inner aspects of the implant was relatively rare.

### Test Material #4

30

These implants triggered strong foreign body reactions but weak fibrotic responses. Implants were surrounded with a thin layer (50 to 150  $\mu\text{m}$  thickness) of fibrous/collagenous capsules, comprised mainly of fibroblasts and collagen fibrils. Large numbers of foreign  
35 body giant cells occurred around and within the implant bodies.

### Test Material #5

Implanted materials prompted strong foreign body reactions  
5 and weak fibrotic responses. The implants were covered by a thin  
fibrous/collagenous capsule (25 to 100  $\mu\text{m}$  thick), comprised  
predominantly of fibroblasts and collagen fibrils. A large number of  
giant cells had invaded the implant body and enveloped the particles  
of test materials.

10

### Test Material #6

Strong foreign body reaction and mild fibrotic responses were  
mediated by the material implants. Implants were surrounded by a  
15 large number of giant cells and some fibroblasts (50 to 150  $\mu\text{m}$   
thick). Cell penetration into this material was marked. A large  
number of giant cells had invaded deeply into the center of the  
material mass and formed many small reticular compartments within  
the implant.

20

### Saline Control

No visible foreign body reaction or fibrotic response was  
found in the tissue as expected. Saline usually disappeared  
25 approximately an hour after injection.

### Conclusions:

Expectably, our negative control (test material #7 - saline  
30 injection) did not cause any visible adverse tissue responses. After  
implantation for 30 days, the test materials triggered widely different  
tissue responses. Qualitatively, the test materials can be divided into  
two distinct groups: 'bioactive' and 'bioinert'. The more bioactive  
materials (test material #2, 4, 5, and 6) triggered stronger tissue  
35 responses (including chronic inflammation, fibrosis, and foreign

body giant cell responses) whereas the bioinert materials (test material #1 and 3) triggered only mild or weak tissue responses.

#### **Example 40**

5

The studies showed that crosslinked polysaccharides (one hyaluronate and two different alginates) are non-immunogenic, which is essential for an implant material.

#### **10 Immunogenic Responses to a Mixture of Polysaccharide Biomaterials in Rabbits**

##### **Study Objective**

15 The objective of this study was to assess the production of antibody to implanted polysaccharide biomaterials in rabbits.

##### **Animals:**

20 Young male adult rabbits (New Zealand White) in good health, weighing approximately 2 Kg, were obtained from Millbrook Farm (Amherst, Massachusetts). Each animal was tattooed with a unique identification number.

##### **25 Test Material and Animal ID Number.**

1. Hyaluronic Acid Beads (45-90  $\mu\text{m}$ ) (animal #1, 2, and 3)
2. Alginate Beads (high mannuronic acid content; 45-90  $\mu\text{m}$ ) (animal #4, 5 and 6).
- 30 3. Alginate Beads (high guluronic acid content; 45- 90  $\mu\text{m}$ ) (animal #7, 8, and 9).

##### **Test Protocol:**

1. Male New Zealand White rabbits (3 animals per test group, total 9 rabbits) were purchased from Millbrook Farms (Amherst, MA) and acclimated for 7 days prior to initiation of this study.
- 5 2. After light anesthesia, each of the nine rabbits was immunized with test materials/Freund complete mixture on day zero (week 0) and then booster shots with test material/Freund incomplete mixture on day 28, 35, 42, 49, and 56 (or week, 4, 5, 6, 7, and 8).
  - 10 2a. Approximately 2.0 ml of the test material mixture/Freund complete (v:v = 1:1) were used for initial injection.
  - 2b. Approximately 2.0 ml of the test material mixture/Freund incomplete (v:v = 1:1) were used for booster shots.
3. The rabbits were monitored for six days after immunization.  
15 Thereafter, weekly inspection of the animals' health, diet and fluid intake were carried out.
4. The blood samples (10 ml) were collected in serum tubes from each animal on week 0 and 10.  
20
5. After clotting at room temperature for two hours, blood samples were centrifuged (500 xg, 10 minutes) to collect serum. Rats were sacrificed by inhalation of CO<sub>2</sub>. The implanted materials and tissues surrounding them were recovered by careful dissection  
25 and fixed with Formalin.
6. The fixed material was embedded *en bloc* in paraffin, sectioned and stained with haematoxylin and eosin.
- 30 Results:
  - A. Material Injected

Approximately 0.5 ml of the test materials was subscapularly administered to both left side and right side of the animal. The weights of injected materials were measured and are listed below.

5

**Weight of Test Materials Implanted In Rats**

	<u>ID#</u>	<u>Left-WT(grams)</u>	<u>Right-WT(grams)</u>
	A-1	0.62	0.59
10	B-1	0.61	0.53
	B-2	0.65	0.59
	C-1	0.58	0.54
	C-2	0.48	0.53
	D-1	0.46	0.55
15	D-2	0.53	0.59
	E-1	0.61	0.53
	E-2	0.49	0.52

**B. Post Injection Animal Care**

20

After implantation, the animals were observed for visible tissue responses around the implantation site. These were recorded at 1, 24, 72, and 144 hours post injection. The injection sites were scored on a scale of 0 to 3 as follows:

25

0 = No reaction

1 = Slight redness and swelling only over implant

2 = Mild redness and swelling over implant and surrounding tissues

30

3 = Severe redness and swelling over implant and surrounding tissues

### Visible Tissue Responses to Implanted Test Materials

5	Test materials	Animal #	The score of tissue responses			
			1 hour	24 hours	72 hours	144 hours
10	A	1	0	0	0	0
	B	1	0	0	0	0
	B	2	0	0	0	0
	C	1	0	0	0	0
	C	2	0	0	0	0
	D	1	0	0	0	0
15	D	2	0	0	0	0
	E	1	0	0	0	0
	E	2	0	0	0	0
	E	2	0	0	0	0

No externally visible inflammatory response or other adverse tissue reactions

#### 20 C. Animal weights:

The weights of animals before and 30 days after implantation were measured. The test materials had no obvious effect on the growth of the test animals.

## D. Antibody Titers:

5	Test Materials	Antibody titration	Means	Gel Surf. Area	Stand. Surf. Area
	1	148,78,100	109 ± 36	16.17cm <sup>2</sup>	0.28 cm <sup>2</sup>
	2	86,32,42	53 ± 29	12.86 cm <sup>2</sup>	0.28 cm <sup>2</sup>
10	3	48,50,108	69 ± 34	14.56 cm <sup>2</sup>	0.28 cm <sup>2</sup>

## Conclusion:

15 Very low antibody titers were seen in animals injected with any of the 3 testing materials. After normalization with surface area, the titer values are completely negligible. Therefore, we conclude that all three testing polysaccharide materials are non-immunogenic.

## 20 Appendix A

Enzyme Linked Immunosorbent Assay For Test Material  
#1, #2, and #3

25 Solution required:

Test serum (diluted 1:10 with PBS)

Pre-immune serum (diluted 1:10 with PBS)

Antigens (test materials #1, #2 and #3)

30 PBS

Washing buffer: PBS/0.05% Tween 20

Blocking solution: PBS/1.0% BSA

Antibody: Protein A conjugated with horseradish peroxidase (HRP)  
(Accurate Chem & Sci #BYA 8605-1) in PBS (10 mM pH 7.4)

35 Citrate buffer: Distilled water 100 ml

1.42 g NaH<sub>2</sub>P0<sub>4</sub> (or 1.63 g of monohydrate)

1.05 g citric acid (1.15 g of monohydrate)

Substrate for horseradish peroxidase (HRP)\*

15 mg o-phenylenediamine (abbreviated as OPD, sigma #P1526)

25 ml citrate buffer

25 ul 30% H2O2

5

\*This substrate must be made up fresh each time prior to the assay.

Equipment required:

- 10 Octavac filter strip system (Fisher)  
96-well strip plates (Fisher #07-200-359)  
filter strip spacer (Fisher #07-200-363)  
Centrifuge  
Standard 96-well plate centrifuge adapter

15

Method:

1. 10 ul of test materials and 90 ul of PBS will be added in 96-well strip plate. This provides about 5 ug of antigen per well. (100 ul PBS will be added to control wells).
- 20
2. Remove solution by centrifugation (500 X g, 10 minutes). 100 ul of PBS will be added to each well. After 10 minute incubation in room temperature, solution will then be removed by centrifugation. Followed with 2 more washes with PBS.
- 25
3. Add 200 ul of blocking solution to each well (including control wells). Incubate at 37°C for one hour.
- 30
4. Wash each well 1X with PBS/Tween 20 as described in step 2, then 2X with PBS.
5. Add series diluted serum (100 ul/well) to each wells using PBS as diluent. Incubate for one hour at 37°C.

35



6. Wash each well 1X with PBS/Tween 20, then 2X with PBS.
7. Add 100 ul of HRP conjugated protein A at 37°C for 1 hours.
- 5 8. Wash each well 1X with PBS/Tween 20, then 2X with PBS.
9. Add 200 ul of substrate (OPD, sigma #P1526) to each well at 37°C for 30 minutes.
- 10 10. Read results (absorbance at 450 nm), compare color with preimmune serum.

Well Number	Treatment Antibody	Antigen
15	1,2 BSA	No (PBS)
	3,4 BSA	Rabbit anti-BSA (1:10)
	5,6 Yes	Control serum (1:10)
	7,8 Yes	Test serum (1:10)
20	9,10 Yes	Test serum (1:20)
	11,12 Yes	Test serum (1:40)

#### Example 41

25 We performed sterility test on hyaluronate microspheres and the results demonstrate that this polysaccharide does not support growth of any bacteria or fungi on its surface or in the material matrix. Moreover, pre-sterilized (i.e., non-autoclaving) gels were also negative for bacteria growth. We conclude that using  
30 polysaccharides such as hyaluronate for implants should limit the occurrence of biomaterial associated infections, a common complication that often results in implant/device failure.

35 We also tested in-vitro cytotoxicity and hemolysis on hyaluronate gels. The results showed the materials are not toxic to the cells, which can be expected since most polysaccharides are extracted from living organisms.

**Purpose:** The purpose of this experiment is to test the sterility of hyaluronate beads both before and after autoclaving.

**5 Procedure:**

1. Submitted one syringe of Pre-Autoclaved hyaluronate beads and one syringe of Post-Autoclaved hyaluronate beads to the microbiology lab for sterility testing.

10

**Note :** Each syringe contains 3 cc of cross-linked hyaluronate beads.

**Results:** Both samples were negative for growth.

**15 Direct Transfer Procedure:**

1. Under a laminar flow hood, expel half the contents of the test syringe into a 25 x 200 mm test tube containing a minimum remaining half into a test tube containing a minimum of 40 ml of sterile Trypticase Soy Broth (TSB).
2. Incubate product samples in FTM at 30-35°C for 14 days, and those in TSB at 20-25°C for 14 days.
- 25 3. After 14 days, check for any signs of turbidity which would indicate bacterial or fungal growth.

**Results:**

- 30 No turbidity was found in the "Before" or "After" samples. This means that no bacteria or fungi were found in the samples. Therefore, the Hyaluronate Before and the Hyaluronate After can be considered sterile.

### Cytotoxicity and Hemolysis Testing

**Purpose:** The purpose of this experiment is to test hyaluronate gel for cytotoxicity and heolysis.

#### Procedure:

1. Prepared syringes, each containing 4 ml of 45-90 micron hyaluronate gel.
2. Submitted the syringes to the Microbiolotu Lab for Cytotoxicity and hemolysis testing.

#### Cytotoxicity (Agar Diffusion Method)

1. Absorbant discs were saturated with the test sample and allowed to drain for approximately five minutes. The discs were transferred to the surface of the agar overlaying the test cell monolayers. The test sample showed no cytotoxic reactivity and met the requirements of the agar diffusion cytotoxicity test.

#### Hemolysis

2. The test procedure was modified slightly due to the viscous nature of the test sample 10 ml of the suspension was transferred to a sterile test tube. 25 ml of sterile saline solution was added to this tube to bring the total volume to 35 ml which was then extracted at 70°C for 24 hours per ML-009. Testing from this point won was per ML-009. The sample had a mean hemolysis value of 1.99%, which meets the requirements of the test.

### Example 42

To qualify as an implant, the implant material must contain very low endotoxin level (<20 EU for one implant device or <5 EU/Kg bodyweight per hour for injection drug, as required by FDA). The raw materials or components of an implant must therefore have a very low level of endotoxin, to avoid expensive depyrogenation steps

We have done endotoxin screening tests on many different polysaccharides raw materials as well as on all crosslinked implantable gels including (1) hydroxypropyl methyl cellulose, (2) inulin, (3) alginates with different formulations, (4) chondroitin sulfate, (5) sodium hyaluronates from different suppliers (7) sephadex with various sizes and different rigidities. The results show either no or very low endotoxin level, which suggests that the polysaccharides are good candidates for implant materials.

### Example 43

We performed in vitro cell culture studies to test whether crosslinked hyaluronate microspheres from two different sources support growth of human fibroblasts. The fibroblast plays an essential role in wound healing and tissue remodeling processes. Briefly, it produces enzymes to digest dead tissue, generate new collagen and other extracellular matrix proteins to reconstruct new tissue, and coordinates many events during inflammation and angiogenesis to ensure smooth remodeling. Implant materials which are compatible with fibroblasts should assist healing. Our studies showed hyaluronate support a normal growth of fibroblasts.

Purpose: The purpose of this experiment is to see if fibroblasts will attach to the Hyaluronate Beads on both Tissue Culture and Non-Tissue Culture Treated Well Plates.

**Procedure:**

- 5       1.    Coated two wells of Hyaluronate Beads from source #1 on  
          both Tissue Culture and Non-Tissue Culture Treated Well  
          Plates.
- 10       2.    Coated two wells of Hyaulronate Beads from source #2 on  
          both Tissue Culture and Non-Tissue Culture Treated Well  
          Plates.
3.    Allowed to dry overnight.
- 15       4.    Placed 100,000 cells in MEM Incomplete Media + L-glutamine  
          in each well.
5.    Allowed to dry overnight.

**Results:**

20

Well Plates were examined under the microscope.

**Tissue Culture Treated Well Plate:**

25

For hyaluronate beads from both sources, the cells attached to the entire surface of the beads and the well.

**Non-Tissue Culture Treated Well Plate:**

30

For hyaluronate beads from both sources, the cells were attached to the beads only.

**Summary:** The cells attached to the beads on both the Tissue Culture Treated and Non-Tissue Culture Treated Well Plates.

### Example 44

We performed extrusion tests on many different crosslinked polysaccharide gels either manually (hand extrusion) or by an Instron machine. By controlling softness and size range of the particles, all polysaccharides can be extruded easily through small gauge needles (e.g., 22 gauge). The elastoviscous property of most polysaccharide materials warrants them as an excellent candidate for injectable implants.

10

We also injected different polysaccharide gels into pig bladders and pig urethras. By comparing the results with collagen injection, we found many polysaccharides provided equal or even better integrity as well as stability (i.e., overall bulking effects) within mucous tissue. We conclude that using crosslinked polysaccharides for soft tissue augmentation is surprisingly effective.

15

#### Procedure:

- 20 1. Placed 0.4 g of beads in a 10 cc syringe.
2. Added 10 ml of PBS to each syringe and mixed thoroughly.
3. Turned each syringe plunger side down and allowed to sit.

- 25 1. Sephadex G-50-50
2. Sephadex G-75-50
3. Sephadex G-200-50
4. Sephadex G-200-120
5. Alginate
6. Chondroitin Sulfate A
- 30 7. Hyaluronate

#### Observations:

Two of the four syringes that were made had separated into a liquid layer and a bead layer.

35

	Sephadex G-50-50	5cc beads	5cc liquid
	Sephadex G-75-50	6cc beads	4cc liquid
	Sephadex G-200-50	10cc beads	
5	Sephadex G-200-120	10cc beads	

#### Procedure:

4. Extruded the liquid layer from the syringes that had  
10 separated.
5. Attached 22 gauge needle.
6. Injected the sample into a pig bladder and a pig urethra.

#### Results:

15 Collagen - Easily extruded through the needle and into the tissues.  
Soft and mushy once inside the tissues. Did not maintain shape.  
Spread out over time. Looks like fat.

20 G200-120 - Easily extruded through the needle and into the tissues.  
Soft once inside the tissues. Maintained shape. Similar to Collagen.

G-50-50 - Easily extruded through the needle and into the tissues.  
Very hard once inside the tissues. Maintained shape very well. Like  
25 a hard tumor.

Alginate - Fairly difficult to extrude into the tissues. Hard once  
inside the tissues. Maintained shape well. Very much like the  
Sephadex 50-50, but not quite as hard.

30 G-200-50 - Easily extruded through the needle and into the tissues.  
Soft inside the tissues. Maintained shape with slight spread overtime.  
Similar to Collagen, but maintained shape better.

Hyaluronate - Easily extruded through the needle and into the tissues. Soft once inside the tissues. Maintained shaped with slight spread overtime. Similar to Collagen, but maintained shape better.

- 5 G-75-50 - Easily extruded through the needle and into the tissues. Hard once inside the tissues. Maintained shape well. Similar to Sephadex G-50-50, but not quite as hard.

- 10 Chondroitin Sulfate A - Easily extruded through the needle and into the tissues. Soft once inside the tissues. Maintained shape with a slight spread over time. Similar to Collagen, but firmer and held shape better.



### Summary of Bladder Injection Data

Sample	Extrudability thru 22 Gauge	Firmness	Stability
Collagen	Easily	Very Soft	Spread out overtime
Sephadex G-50-50	Easily	Very Hard	Maintained shape
Sephadex G-75-50	Easily	Hard	Maintained shape
Sephadex G-200-50	Easily	Soft	Slight spread overtime
Sephadex G-200-120	Easily	Soft	Maintained shape
Alginate	Difficult	Hard	Maintained shape
Hyaluronate	Easily	Soft	Slight spread overtime
Chondroitin Sulfate A	Easily	Soft	Slight spread overtime

#### 5 Samples Ranked from Hardest to Softest

1. Sephadex G-50-50
2. Alginate
3. Sephadex G-75-50
- 10 4. Hyaluronate
5. Chondroitin Sulfate A
6. Collagen\*
6. Sephadex G-200-120\*
6. Sephadex G-200-50\*

15

\*All very similar

#### Procedure:

- 20 7. Cut each injection site open to see the texture of the beads once inside the tissues.

Observations: When the injection site was cut open with a scalpel, each sample came out in a paste-like gel.

25

Purpose: To inject beads into a pig bladder and a pig urethra while observing the ease of extrudability and whether or not the beads remain in place once inside the bladder and urethra tissues.

**Procedure:**

1. Placed 0.4 g of beads in a 10 cc syringe.
2. Added 10 ml of PBS to each syringe and mixed thoroughly.
- 5 3. Turned each syringe plunger side down and allowed to sit overnight.

The four syringes made are as follows:

- 10 1. Sephadex G-50-50
  2. Sephadex G-200-50
  3. Sephadex G-200-120
  4. Sepharose CL-2B-300
- 
- 15 4. Extruded the liquid layer from each of the four syringes.
  5. Attached 22 gauge needle.
  6. Injected into pig bladder and urethra.
  7. Additionally collagen was injected into the pig bladder and urethra also.

20

**Results:**

Collagen - Easily extruded through the needle and into the tissues. Remained in place once inside the tissues. Formed a soft and mushy  
25 bump where injected. It was also very soft and pliable.

The first injection into the bladder resulted in a bleb (blister like pustule). This was because the injection was made too close to the surface. The second injection into the bladder was deep enough into  
30 the tissues so as not to cause a bleb, but a visible lump never appeared in the tissue. When injected into the urethra another bleb formed. Also the collagen would start shooting out of hole in which the needle was inserted.

G-50-50 Easily extruded into the bladder and urethra. Stayed in place once injected into the tissues. Formed an extremely hard bump where injected. The bump could not be moved around or squeezed.

5    **Sephadex G-200-50**

Easily extruded into the tissues. Remained in place at first, but slowly began to dissipate. At first formed a firm bump, but did not hold shape with time. Even though it was firm it was not nearly as  
10    firm as the Sephadex G-50-50 and with time it became as soft and pliable as the collagen.

**Sephadex G-200-120**

15            Easily extruded into the tissues.  
              Remained in place after injection.  
              Formed a hard ball inside the tissue. This ball was  
              harder than the G-200-50, but not as hard as the G-50-  
              50.

20            First injection into the urethra was deep enough to form  
              a visible knot without bleeding. The knot retained its  
              shape well.

25    **Chondroitin Sulfate A -**

Easily extruded into the tissues.  
Remained in place once inside the tissues.  
Formed a firm knot at first, but lost its firmness  
30    overtime.

## INJECTIONS

Sample	Extrudability thru 22 Gauge	Stability	Firmness
Collagen	Easily	Remained in place	Soft and pliable
Sephadex G-50-50	Easily	Remained in place	Very hard knot
Sephadex G-200-50	Easily	Dissipated with time	Firm at first, but soft overtime
Sephadex G-200 -120	Easily	Remained in place	Hard knot formed
Hyaluronte (Lifecore)	Unable in tissues	n/a	n/a
Chondroitin Sulfate A	Easily	Remained in place	Firm knot formed
Alginate (Sigma)	Easily	Remained in place	Firm at first, but soft overtime

- 5 Purpose: To swell Sephadex beads and then test their extrudability through a 22G1 needle on the Instron machine.

## Procedure:

- 10 1. Placed 0.5 g of beads in small beaker.  
 2. Added 30 ml of PBS to the beads.  
 3. Allowed beads to swell overnight.  
 The following beads were used:  
     Sephadex G-50-50  
 15      Sephadex G-75-50  
     Sephadex G-200-50  
     Sephadex G-200-120  
 4. Packed the beads in 3 cc syringes. Three syringes were made for each type of beads.  
 20 5. Tested each syringe on the Instron machine using a 22G1 needle. The average load at maximum load and the average load between limits were was 14.41 lbs. and 0.6584 lbs. for the G-50-50; 5.632 and 0.5047 for G-75-50; 0.9315 and 0.3349 for G-200-50; and 0.9450 and 0.4018 for G-200-120.

The purpose of this experiment was to test the amount of force needed to extrude Pre-Autoclaved Hyaluronate Beads and Post-Autoclaved Hyaluronate Beads through a syringe.

5   **Procedure:**

Tested each syringe on the Instron using a 22G1 needle.

Results:

10   **Pre-Autoclaved Hyaluronate Beads**

Maximum Load: 4.333 lbs.

Average Load Between Limits 1: 0.3037 lbs.

(This is the average load from start to finish).

15

**Post-Autoclaved Hyaluronate Beads**

Maximum Load: 4.327 lbs.

Average Load Between Limits 1: 0.3010 lbs.

20

(This is the average load from start to finish).

**Summary:**

25   Autoclaving has no effect on the extrudability of the Hyaluronate Beads.

30   Although the present invention has been described in detail with respect to various embodiments thereof, it should be clearly understood that the same is by way of example and illustration only and is not to be taken by way of limitation, the spirit and scope of the present invention being limited only by the terms of the appended claims.

What is Claimed is:

1. A process for the preparation of crosslinked water-soluble polymer particles comprising,  
5 combining an aqueous polymer solution comprising a water-soluble polymer and an aqueous medium, and an oil medium so as to form an emulsion of droplets of the water-soluble polymer; and  
10 adding to the emulsion a crosslinking agent capable of crosslinking the water-soluble polymer so as to form crosslinked water-soluble polymer particles.
2. The process of Claim 1, wherein an emulsifier is added to the oil medium.  
15
3. The process of Claim 2, wherein the emulsifier is hexadecyl sodium phthalate, sorbitan monostearate, or a metal soap.
- 20 4. The process of Claim 1, further comprising inverting the polymer particle-containing emulsion in an excess of water to provide an aqueous dispersion of crosslinked water-soluble polymer particles.
- 25 5. The process of Claim 4, further comprising recovering the crosslinked water-soluble polymer particles.
6. The process of Claim 1, wherein the ratio of the weight of the water-soluble polymer to that of the crosslinking agent is from about 0.1 to about 10.  
30
7. The process of Claim 1, wherein the ratio of the weight of the water-soluble polymer to that of the crosslinking agent is selected so as to yield particles that are less than 212  
35 micrometers in diameters.

8. The process of Claim 1, wherein the ratio of the weight of the water-soluble polymer to the weight of the crosslinking agent is selected so as to yield particles that are less than  
5 150 micrometers in diameters.

9. The process of Claim 1, wherein the pH of the aqueous polymer solution is adjusted above or below pH 7 so as to reduce the rate of the reaction of the crosslinking agent with the  
10 water-soluble polymer, and wherein after the emulsion is formed the pH is adjusted to approximately pH 7.

10. The process of Claim 1, wherein the water-soluble polymer is sodium alginate, poly(N-vinyl pyrrolidone),  
15 methyl cellulose, poly(vinyl alcohol), chondroitin sulfate, hyaluronic acid, bovine serum albumin, human gamma globulin or combinations thereof, and wherein the crosslinking agent is pentaerythritol-tris-[beta (N-aziridiny)-propionate], divinylsulfone, glutaraldehyde, p-toluene sulfonic acid, carbodiimides, diepoxides, or ammonium  
20 persulfate, and wherein the oil medium is toluene, o-xylene, or isooctane.

11. A crosslinked water-soluble polymer particle preparation wherein the particles are substantially  
25 homogeneous in size, wherein the particles are less than 212 nm in diameter, and wherein at least 80% of the particles are spherical.

12. The polymer particle of Claim 11, wherein the size of the polymer particles is less than about 150 micrometers  
30 in diameter.

13. The polymer particle of Claim 11, wherein the crosslinking is provided by covalent bonds.

14. The polymer particle of Claim 11, wherein the water-soluble polymer comprises a polysaccharide.

15. The polymer particle of Claim 14, wherein  
5 the polysaccharide is hyaluronic acid, sodium alginate, chondroitin sulfate, celluloses, chitin, chitosan, agarose, dextrans, xanthans, dermatan sulfate, keratin sulfate, amylose, emulsan, gellan, curdlan, amylose, carrageenans, amylopectin, dextrans, glycogen, starch, heparin sulfate, limit dextrans or fragments thereof.

10

16. The polymer particle of Claim 11, wherein the polymer particle is formed by the process of Claim 1.

17. The polymer particle of Claim 11, further  
15 comprising an aqueous medium to form an aqueous dispersion comprising a plurality of crosslinked water-soluble polymer particles.

18. The polymer particle of Claim 17, wherein  
20 the aqueous dispersion of polymer particles is extrudable through a 20 gauge hypodermic syringe needle.

19. The polymer particle of Claim 11, further comprising a bioactive agent.

25

20. A method comprising, administering to a patient in need of treatment an aqueous suspension of the water-soluble polymer particles of Claim 11.

21. The method of Claim 20, wherein the  
30 aqueous suspension of the water-soluble polymer particles comprises a polysaccharide.



22. The method of Claim 21, wherein the polysaccharide is hyaluronic acid, sodium alginate, chondroitin sulfate, celluloses, chitin, chitosan, agarose, dextrans, xanthans, dermatan sulfate, keratin sulfate, amylose, emulsan, gellan, curdlan, amylose, carrageenans, amylopectin, dextrans, glycogen, starch, heparin sulfate, limit dextrans or fragments thereof

23. The method of Claim 20, wherein the aqueous suspension of water-soluble polymer particles is administered to a patient in need of soft tissue augmentation.

24. The method of Claim 23, wherein the aqueous suspension of water-soluble polymer particles is administered to a patient to treat a defect selected from the group consisting of urinary incontinence, vesicoureteral reflux, glottic insufficiency, gastroesophageal reflux, and skin defects.

25. The method of Claim 20, wherein the aqueous suspension of water-soluble polymer particles is administered to a patient to serve as a scaffold material for wound healing.

26. The method of Claim 20, wherein the polymer particles further comprise a bioactive agent.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/10249

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C08J3/26 A61L31/00 A61L27/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C08J C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 124 705 (U.S.E.ROTHMAN ET AL.) 7 November 1978 see claims 2,9,10,17,24,25 see column 5, line 18-36 see column 8, line 3-14 see column 8, line 31-52 see column 9, line 25-62 see examples 1,2,9	1,2,6-9, 11-22,26
X	WO,A,91 01721 (E. VANZO) 21 February 1991 see claims 1-3 see page 2, line 27-31; figures 1-7 see examples 1,5 --- -/--	1,2,6-9, 11-15

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

4 November 1996

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/10249

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 256 293 (MITSUBISHI CHEMICAL INDUSTRIES LIMITED) 24 February 1988 see claims 1-4,6-9 see page 2, line 11-33 see page 4, line 9-17 see examples 1-5 ---	1-3,6-13
X	EP,A,0 555 980 (NISSHINBO INDUSTRIES, INC) 18 August 1993 see claims 4-8 see figure 1; example 1 ---	11,12, 14,15
A	DATABASE WPI Week 9028 Derwent Publications Ltd., London, GB; AN 90-213152 XP002017566 & JP,A,02 142 701 (NORINSHO ET AL) , 31 May 1990 see abstract ---	11,14, 15,17,18
A	GB,A,2 151 244 (BIOMATRIX INC.) 17 July 1985 see claim 1 see example 10 ---	11,13-15
A	EP,A,0 507 604 (ETHICON INC. ) 7 October 1992 see claims 11-13 -----	11-20

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 10249

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
**ALTHOUGH CLAIMS 20-26 ARE DIRECTED TO A METHOD OF TREATMENT OF THE HUMAN/  
ANIMAL BODY THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED  
EFFECTS OF THE COMPOSITION**
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/10249

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/10249

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